GREEN CHEMISTRY AND CIRCULAR ECONOMY AS ALTERNATIVE STRATEGIES FOR THE TRADITIONAL LEATHER MANUFACTURING INDUSTRY

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RIASSUNTO

L'industria conciaria italiana copre circa il 19% della produzione mondiale di pellame, ricoprendo un ruolo fondamentale non solo nella rappresentazione del concetto del *Made in Italy* nel mondo ma anche nel panorama economico nazionale.

Minacciata dalla sempre più ampia diffusione di materiali alternativi alla pelle, l'industria conciaria ha sviluppato, nel corso degli anni, tecnologie sempre più innovative per rispettare gli standard di qualità imposti dal mercato e aumentare la sua competitività, trascurando, soprattutto in passato, l'impatto ambientale dei processi di lavorazione adottati. Si sono così affermate nella filiera, fasi di processo che impiegano sostanze non solo inquinanti ma anche potenzialmente tossiche. Ne consegue che, nonostante la lavorazione del pellame sia basata sul recupero e la valorizzazione di uno scarto industriale (la pelle animale derivante dalla macellazione) in un quadro di economia circolare, questa attività industriale sia, ad oggi, etichettata come una delle più inquinanti svolte dall'uomo.

L'attenzione rivolta alla sostenibilità ambientale diffusa nell'ultima decade ha, però, stimolato un cambiamento di direzione da parte dell'industria conciaria, spingendo i ricercatori allo sviluppo di nuove tecnologie in grado di garantire sia le caratteristiche finali del prodotto, richieste dal mercato, che la riduzione dell'impiego di agenti inquinanti. In questa prospettiva sono stati sviluppati metodi di concia alternativi al cromo, uno dei principali responsabili dell'accezione negativa dell'industria conciaria, e sono stati introdotti, nella filiera produttiva, nuovi approcci biotecnologici, basati sull'impiego di enzimi, sostituendo efficacemente i diversi processi della pre-concia, quali la depilazione, la calcinazione e il bagno.

Sebbene l'adozione di processi eco-friendly riduca notevolmente la concentrazione degli inquinanti nelle acque reflue, l'eco-sostenibilità dell'industria conciaria è, tuttavia, ancora minacciata dall'ingente quantità di rifiuti solidi prodotti. Si stima, infatti, che per ogni tonnellata di pelle animale trattata, solo il 20% sia convertito in prodotto e il restante 80% sia smaltito come rifiuto solido, dando luogo alla produzione di circa 8.5 milioni di tonnellate di rifiuti solidi per anno. Inoltre, a seconda della fase di processo durante la quale sono generati (pre-concia, concia o post-concia), questi scarti possono contenere agenti chimici che li rendono difficilmente degradabili; sono, infatti, classificati come rifiuti speciali (secondo il Catalogo Europeo dei Rifiuti), il cui smaltimento costa circa 1,2 miliardi di euro per anno.

È evidente quindi che, gravando non solo sul piano economico ma anche su quello ambientale, il recupero e la valorizzazione di questi

scarti sia alla base della transizione eco-sostenibile dell'industria conciaria.

In questo scenario s'inquadra il presente progetto di dottorato, svolto in collaborazione tra l'Università di Napoli Federico II, la Stazione Sperimentale per l'industria delle Pelli e le materie concianti (SSIP) e BioPox srl, e che mira allo sviluppo di nuovi processi per il recupero e la valorizzazione degli scarti conciati dell'industria del pellame, attraverso approcci biotecnologici per l'estrazione, il recupero e la valorizzazione del collagene. Il progetto rientra nell'ambito di un Dottorato Innovativo con caratterizzazione Industriale, supportato dal Ministero dell'Università e della Ricerca con il Programma Operativo Nazionale (PON) Ricerca e Innovazione 2014-2020.

Il lavoro si è articolato nelle seguenti fasi:

Recupero degli scarti solidi dell'industria conciaria: estrazione e caratterizzazione del collagene

Gli scarti solidi dell'industria conciaria (principalmente costituiti da pelli grezze e semilavorati) si sono rivelati essere una potenziale fonte di prodotti ad alto valore aggiunto come oli, agenti di riconcia e proteine, in particolare collagene.

Il collagene costituisce circa il 90% della frazione proteica presente nella pelle animale e, svolgendo in natura funzioni di protezione e sostegno, è particolarmente versatile nelle applicazioni industriali: esso viene, infatti, coinvolto nelle formulazioni di cosmetici e fertilizzanti, nello sviluppo di matrici per la crescita cellulare e nella ricostruzione di ossa e cartilagini. L'interesse industriale verso questa proteina ha spinto allo sviluppo di metodologie per la sua estrazione dai rifiuti solidi del pellame. Gli scarti provenienti dai processi di pre-concia, ad esempio, sono da tempo efficacemente trattati con approcci chimici e biotecnologici per il recupero di collagene, grassi e cheratina.

Ponendo l'attenzione sui rifiuti prodotti dai processi di concia, invece, la complessità della sfida tecnologica aumenta: durante la concia, la pelle animale è trattata con agenti chimici in grado di stabilire nuovi legami tra le fibre di collagene, convertendo la pelle, naturalmente soggetta alla degradazione microbica, in un materiale non putrescibile. Ne consegue che il collagene presente sia stabilizzato e poco disponibile all'azione di agenti esterni. Inoltre, l'ampia varietà di tipologie di concia genera eterogeneità degli scarti, rendendo complesso lo sviluppo di metodi per la loro valorizzazione.

Nell'ultima decade, gli approcci studiati per il recupero dei rifiuti conciati si sono basati principalmente sull'idrolisi chimica, utilizzando

agenti acidi o alcalini in grado di dissolvere la matrice della pelle e rilasciare il collagene. Questo tipo di estrazione comporta un'idrolisi molto spinta, causando non solo la degradazione della pelle-scarto, ma anche quella del collagene, limitando quindi fortemente il suo riutilizzo che viene ristretto a settori a basso valore aggiunto quali l'uso come additivo dei fertilizzanti nell'agricoltura. Va inoltre considerato che l'impiego di significative quantità di agenti chimici per l'estrazione di collagene se, da un lato, ne permette il recupero, seppure inficiandone la qualità, dall'altro comporta un aumento della concentrazione degli inquinanti nelle acque reflue.
Nell'ambito di questo progetto, sono stati sviluppati nuovi metodi di estrazione di collagene dai rifiuti di pelle conciata, sostituendo l'idrolisi chimica con quella enzimatica. In questo modo, la reazione di idrolisi può essere finemente controllata, ottenendo una resa maggiore non solo in termini di contenuto proteico estratto, ma anche in termini di qualità del collagene recuperato: è stato dimostrato, infatti, che il collagene ottenuto conserva integra la sua struttura. L'impiego degli enzimi per il trattamento degli scarti della lavorazione permette di ridurre i tempi del processo, risultando circa il 50% meno dispendioso dei processi chimici tradizionali di estrazione. Il processo enzimatico, inoltre, consente una riduzione di almeno 10 volte la quantità di agenti chimici utilizzati, risultando quindi un processo più vantaggioso anche dal punto di vista ambientale.
Con lo scopo di applicare il collagene estratto nuovamente nella filiera produttiva del pellame, mirando quindi non solo allo sviluppo di tecnologie sostenibili ma anche alla circolarità del flusso produttivo dell'industria conciaria, è stato sviluppato, inoltre, un processo di cross-linking enzimatico del collagene con la caseina per rendere il collagene estratto più resistente allo stress termico.

Valorizzazione degli scarti solidi dell'industria conciaria: applicazione del collagene estratto

Il processo di produzione del pellame è lungo e complesso: la pelle animale è sottoposta a una serie di procedure chimiche e meccaniche per convertire un materiale putrescibile in cuoio. La conversione della pelle in cuoio è suddivisa in tre fasi principali: la pre-concia, la concia e la post-concia. La quasi totalità delle acque reflue e degli scarti solidi prodotti dall'industria conciaria proviene dalle prime due fasi della lavorazione; sebbene l'enorme quantità di rifiuti prodotti causi un'urgente problematica ambientale, la composizione di questi scarti li rende un'appetibile risorsa di composti ad alto valore aggiunto, stimolando lo sviluppo di processi il recupero.

Contrariamente, la post-concia vede una percentuale minima di produzione di scarti, costituiti da cuoio rifinito e reflui ricchi di inquinanti e composti potenzialmente tossici. Ne consegue che piuttosto che agire a valle della produzione di questi scarti, sia necessario, dal punto di vista della transizione verso una produzione più pulita, agire all'origine della produzione degli scarti e sostituire gli agenti chimici inquinanti utili alla post-concia, senza inficiare sulla qualità del prodotto finale.
In questo progetto, l'attenzione è stata rivolta a due fasi cruciali del processo di post-concia: la riconcia e la rifinizione.

Applicazione del collagene estratto come agente di riempimento
La fase di riconcia è fondamentale per aumentare la qualità dei prodotti, uniformando la matrice della pelle. Durante questa fase, le imperfezioni presenti sulla pelle, quali rughe e aree vuote, sono minimizzate dall'introduzione di agenti di riempimento. L'agente di riempimento ideale deve essere compatibile con la pelle, ben solubile in acqua, capace di penetrare nella matrice del cuoio e in grado di riempire in modo omogeneo gli spazi vuoti tra le fibre.
Nell'ambito di questo progetto, grazie alle sue caratteristiche, il collagene estratto si è rivelato essere un ottimo candidato per la fase di riempimento della pelle. È stato, quindi, riprogettato il processo di riconcia, introducendo la reazione di cross-linking enzimatico tra il collagene e la caseina come fase di riempimento, sostituendo completamente gli agenti di riempimento utilizzati tradizionalmente dall'industria conciaria e proponendo un nuovo processo di riconcia più eco-sostenibile,
La pelle trattata è stata sottoposta ad analisi microscopiche e fisiche ed è stato dimostrato che il collagene, data la sua naturale compatibilità con la pelle, è in grado non solo di penetrare la matrice del cuoio, ma anche di riempire in maniera omogenea gli spazi vuoti tra le fibre, risultando in un prodotto più uniforme e piacevole al tatto.
Il nuovo processo di riconcia valorizza le pelli di bassa qualità, caratterizzate da rughe e aree vuote e quindi destinate alla produzione di articoli meno esclusivi e con meno valore aggiunto, sia dal punto di vista qualitativo che economico, aumentandone il valore merceologico di circa il 30%.

Applicazione del collagene estratto come agente di rifinizione
La fase di rifinizione è responsabile dell'aspetto finale della pelle. Questa fase è anche quella che maggiormente utilizza sostanze chimiche inquinanti come composti organici volatili, cross-linker potenzialmente tossici e resine sintetiche difficilmente biodegradabili.

In questa prospettiva, lo sviluppo di una formulazione di finitura in grado di conferire alla pelle la durevolezza e la qualità richieste dal mercato e, allo stesso tempo, che segua una direzione eco-sostenibile è una delle sfide più impegnative per l'innovazione dell'industria della pelle.

L'ultima parte di questo progetto ha proposto l'applicazione del collagene estratto come componente, insieme alla caseina e al cross-linker enzimatico, di una nuova formulazione di rifinizione, proponendosi come sostitutivo delle tradizionali resine poliuretaniche e acriliche.

La formulazione contenente collagene è stata preparata come film per analizzarne le proprietà e poi applicata sulla pelle. Una volta applicata la formulazione sviluppata, la pelle rifinita è stata sottoposta a prove meccaniche di resistenza del colore e confrontata con una pelle rifinita con una formulazione tradizionale.

Al fine di ottenere le stesse proprietà della pelle trattata con la formulazione di collagene e quella trattata con la formulazione tradizionale, la prima è stata ottimizzata in termini di quantità di enzima, di collagene e di caseina.

La pelle trattata con la formulazione di collagene così ottimizzata ha mostrato le stesse proprietà di resistenza del colore all'abrasione, alla luce artificiale e alla goccia d'acqua delle tradizionali rifinizioni basate su poliuretani e acrilati. Il prodotto ottenuto ha inoltre il vantaggio di risultare almeno 10 volte più velocemente biodegradabile.

Il presente progetto di dottorato ha contribuito allo sviluppo di nuovi approcci biotecnologici per la transizione dell'industria conciaria verso una produzione verde e circolare. I suoi obiettivi sono stati raggiunti attraverso lo sviluppo di nuove procedure per il recupero del collagene dagli scarti solidi e la sua applicazione nella lavorazione della pelle come agente di riempimento.

SUMMARY

During the last decades, the increasing social and political attention to environmental sustainability has driven the industrial sectors towards the development of cleaner and closed-loop productions. Leather industry is one of the most fitting instances of the green transition. Although it is originally based on the principles of Circular Economy, being leather the result of the recovery and valorization of a by-product of the meat industry, nowadays this industrial sector is labeled as one of the most polluting, due to the high polluting procedures and the huge amounts of wastes produced.

The green transition of tanning industry is, therefore, aiming to the development of alternative biotechnological approaches to traditional methods and focusing on the recovery of wastes produced.

In this scenario and in a Circular Economy perspective, the present PhD project has been defined. The objectives of the project are related to the development of green strategies for tanning processes, by *i)* recovering leather solid wastes through the extraction of collagen and *ii)* applying the extracted collagen in leather processing, replacing the polluting chemicals that are conventionally used.

For the first part of the project, four different kind of tanned solid wastes have been treated to extract collagen. Two cost-effective enzymatic extraction methods were developed and optimized in terms of amounts of chemicals, reaction time and extraction yields. The extracted collagen was characterized and it results that the developed extraction methods allow extracting high quality collagen.

To employ back collagen in the leather processing, an enzymatic-mediated cross-linking reaction between collagen and casein was set up *ex situ* and optimized on the basis of the percentage of high molecular weight moieties formed.

In the second part of the project the extracted collagen has been applied as filling agent for low quality leather and as finishing agent during the last part of leather processing.

Regarding the filling, the optimized cross-linking reaction was exploited *in situ,* re-designing the re-tanning process. It results that leather treated with collagen appeared more uniform, well-filled and more pleasure at the touch than the leather treated with conventional re-tanning process.

Finally, the extracted collagen was used as component of finishing formulation, allowing to develop an eco-friendly finishing system and to obtain treated leather with characteristics comparable to those of leather treated with the top-of-the-range finishing formulation.

INTRODUCTION

Introduction

During the last decades, the idea of sustainable development has spread worldwide: industrial processes, originally based on chemical methods, have been gradually replaced by eco-friendly strategies (Kalmykova et al., 2018). In this scenario, the concept of Circular Economy, described by its three main principles: reduce, reuse and recycle (Heshmati, 2017), has emerged as a sustainable development strategy, harmonizing economic growth and environmental protection and promoting the transition towards eco-sustainability through a better use of resources (Velenturf and Purnell, 2021).

Along with Circular Economy, Green Chemistry is a fundamental tool to drive the transition to circularity (Loste et al., 2020): it is, indeed, the use of chemical and biotechnological methodologies to reduce the use of raw materials and the generation of wastes that result to be hazardous to human health and environment (Horváth and Anastas, 2007).

Circular Economy and Green Chemistry are, therefore, the new tools that industry employs to develop economically and environmentally sustainable technological innovations (Anastas and Williamson, 1996).

One of the most representative examples of transition towards circularity is the case of leather industry.

Leather industry

Leather industry is an ancient and profitable manufacturing sector that produces (approximately US$ 100 billion per year of global trade value), (Maina et al., 2019; Pringle et al., 2016), a variety of goods by the processing and valorization of the industrial wastes derived from meat production (Sivaram and Barik, 2018).

Notwithstanding its processing is based on one of the key principles of circular economy, leather industry is currently labeled as one of the most polluting industrial activities (Nazer et al., 2006). Threatened by the increasing spread of alternative materials to leather, the tanning industry has developed, over the years, more and more innovative technologies to meet the quality standards imposed by the market to increase its competitiveness (Ollé et al., 2009a), neglecting the environmental impact of the working processes adopted. Process phases, which employ not only polluting but also potentially toxic substances, have therefore established themselves in the supply chain (Kanagaraj et al., 2006). In addition to the use of harmful substances, there is also a huge generation of wastewaters and solid wastes during leather manufacturing processes.

If, on one hand, leather industry adopted biotechnological approaches to replace several traditional steps of the industrial process, such as the use of proteases during the unhairing (Dettmer et al., 2012; Sivasubramanian et al., 2008), lipases for the degreasing (Kanagaraj et al., 2015) and collagenases and laccases for dyeing (Dettmer et al., 2013; Pezzella et al., 2016), on the other hand the disposal of wastes still remains a critical issue that needs to be solved.

Leather manufacturing process

Leather processing consists in chemical and mechanical operations that aim to convert raw hides and skins, susceptible to microbial degradation, into a durable and pleasant material (Sizeland et al., 2015); it is divided into three main steps: pre-tanning, tanning and post-tanning operations (Maina et al., 2019).

Pre-tanning processes are the first step of leather manufacturing and comprises several operations for the cleavage and preparation of raw hides and skins (Muralidharan et al., 2022): at the end of pre-tanning, raw hides and skins are deprived of blood, fats, proteins (except collagen) and salts (used for the conservation), (Ouertani et al., 2021).

Tanning is the most significant process for the conversion of raw hides in leather (China et al., 2020). During this step, indeed, the collagen fibers of the starting material are stabilized against the microbial attack through covalent or hydrogen bonds with the tanning agents (Wu et al., 2020).

Finally, post-tanning operations are responsible of the final aspect of the products (Hansen et al., 2020). These processes consist not only in the use of dyes, but also in the application of resins, fats and oils to confer to the leather the required aesthetic characteristics, such as uniform appearance and softness (Villaseñor-Basulto et al., 2022).

Each of these operations requires several inputs, such as water and reactants, which are converted in outputs that consist not only in semi-processed skins or leather, but also in wastewaters and solid wastes (Thanikaivelan et al., 2005).

Leather wastes

The production of leather leads to the generation of great volumes of wastewaters and huge amounts of solid wastes (Dixit et al., 2015): for each ton of raw hides, approximately 30-35 m^3 of wastewaters and 800 kg of solid wastes are disposed, generating around 8.5 million tons of solid wastes per year (Ozgunay et al., 2007). On the basis of the phase of the process during which they are produced, wastewaters and solid wastes can contain harmful and/or valuable substances (Sathish et al., 2019).

As shown in Figure 1, pre-tanning operations are responsible for the higher percentage of wastewaters and solid wastes generated during the whole process of conversion of raw hides in leather. Both pre-tanning and tanning processes, which account nearly for around 90% of the total pollution of leather processing (Thanikaivelan et al., 2005), produce wastes rich in high-added value compounds. Instead post-tanning operations if, on one hand, generate low percentage of wastes, on the other hand contain few valuable chemicals (Ouertani et al., 2021) (Fig. 2). Therefore, the attention for the development of recovery strategies have been mainly focused on the wastes produced during the first steps of leather processing.

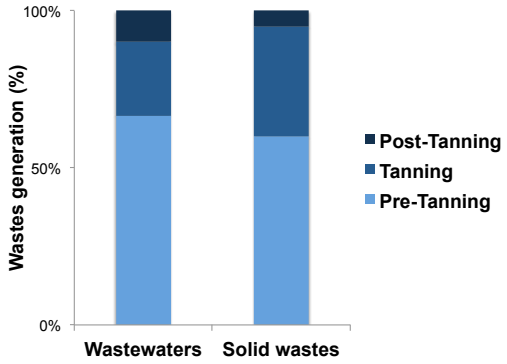

Figure 1. Percentage of wastewaters and solid wastes produced during pre-tanning, tanning and post-tanning operations

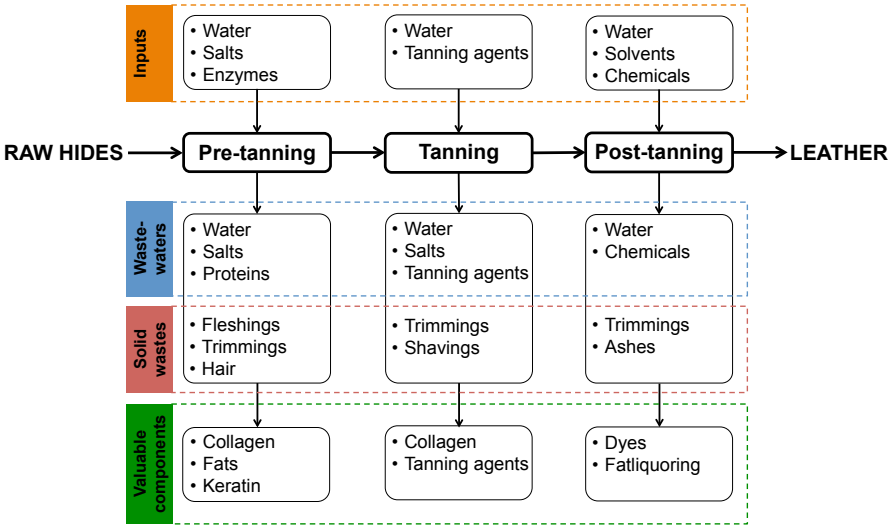

Figure 2. Workflow, inputs and outputs of leather manufacturing process

From wastes to resource

Leather solid wastes are usually disposed in the most common and simplest way, *i.e.* by discharging them on specific land sites (Rigueto et al., 2020). Tanneries have adopted conventional wastes recovery processes, based on chemical and physical methods that, however, are high energy and time consuming and cause severe pollution (Thanikaivelan et al., 2005). The even more increasing attention on environmental sustainability, which is spreading during the last decades, has encouraged researchers in developing clean methods, based on biotechnological strategies, for the recovery and valorization of leather wastes converting them into valuable products. Moreover, biotechnological strategies require, compared to conventional methods, milder operative conditions, allowing not only energy and time saving, but also a significant decrease of pollution (Puhazhselvan et al., 2017).

One of the most appealing recoverable products is collagen. Collagen is a versatile fibrous protein, which composes the extracellular matrix in mammals and marine organisms (Silvipriya et al., 2015). Due to its structure and function, collagen finds numerous applications in several industrial fields (Abraham et al., 2008; Schmidt et al., 2016; Sionkowska et al., 2017).

Aim of the thesis

This PhD project aimed to develop feasible strategies for leather industry, according to the principles of Circular Economy and Green Chemistry. It is divided into two main objectives: the first one is focused on the conversion of tanned solid wastes into collagen sources (chapter 1); while the second objective is focused on the application of recovered collagen again in leather processing replacing polluting chemicals (chapter 2).

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Introduction

CHAPTER 1 - RECOVERY OF LEATHER SOLID WASTES: EXTRACTION AND CHARACTERIZATION OF COLLAGEN

Leather solid wastes are classified, according to the process that produces them, in untanned, tanned and post-tanned wastes. The recovery of tanned wastes represents the most challenging goal. First of all, the high heterogeneity of tanning agents makes these wastes difficult to treat, the stabilization of collagen fibers makes difficult collagen extraction and, finally, the nature of tanning agents may classify these wastes as hazardous (Jayanthi et al., 2019; Sivaram and Barik, 2018; Wu et al., 2020).
The conventional methods for collagen extraction from tanned wastes employ high quantity of alkaline or acid substances that degrade leather and release collagen peptides (Ding et al., 2015). In the last decades, research has been focused on the development of new effective and sustainable methods for collagen extraction, focusing on the quality of collagen, the versatility of the methods and the reduction of chemicals. For this purposes, the development of enzymatic hydrolysis can be the most fitting strategy, allowing to finely tune the hydrolysis degree and to avoid the use of hazardous substances. The critical issue of this approach is related to the complexity of these wastes that requires an integration, to the enzymatic reaction, of several procedures that must be eco-friendly to not affect the sustainability of the process (Scopel et al., 2019).
This chapter describes new collagen extraction procedures that allow *i)* to recover high quality collagen; *ii)* to reduce the amount of chemicals; *iii)* to obtain higher extraction yields compared to conventional methods; and *iv)* to reduce the extraction cost per kilogram of recovered collagen.

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Leather industry towards circular economy: enzymatic extraction of collagen from tanned wastes

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Abstract

The environmental impact of tanning production involves significant issues, due to the volumes of wastewater and the amounts of solid wastes that are produced during the process. In order to reduce its environmental impact, leather industry is moving towards cleaner production methods mainly focusing on recovery and valorization of tanning wastes. Leather solid wastes are, indeed, an appealing collagen source, which has a wide range of applications depending on its structural integrity. To take advantage of its physiological function, indeed, collagen needs to maintain its structure.

The recovery of collagen from leather wastes has been extensively studied, but still presents several critical issues. On one hand, the cost-effective chemical extraction methods employ huge amount of alkaline or acid compounds that affect the environmental sustainability of the process. On the other hand, enzymatic extraction methods reported until now are more expensive respect to the traditional chemical ones, provide low yields of recovered collagen and require long reaction time. Tanning industries, moreover, still adopt both approaches recovering, from its wastes, only collagenous products, *i.e.* small collagen polypeptides or gelatin.

We suggest new cost-effective enzymatic methods for collagen extraction from leather solid wastes, which allow reducing the use of chemicals and reaction time and preserving collagen quality. Respect to collagenous products, high quality collagen finds a wider range of high added-value applications, including its re-utilization in leather manufacturing processing.

Collagen, extracted via the new enzymatic methods, was characterized for protein, metal and polyphenols content; furthermore, molecular weight distribution of the extracted proteins was evaluated to determine the quality and the structural integrity of collagen. The preservation of the structure of collagen after enzymatic extraction was also assessed through infrared spectroscopy analysis.
Extracted collagen was enzymatically cross-linked with casein to verify its applicability as filling agent. The enzymatic cross-linking process was optimized in terms of degree of cross-linking and of molecular weights of the moieties formed, reaching up to 80% of cross-linked collagen.

Keywords: Green Chemistry, Circular Economy, collagen, leather industry

Introduction

Notwithstanding leather manufacturing seems originally and intrinsically based on circular economy concept (Thanikaivelan et al., 2004), leather industry is considered one of the most polluting sectors (Rosu et al., 2018), generating around 4 million tons of solid wastes per year (Fela et al., 2011). The economic and environmental impact related to the disposal and management of leather solid wastes is huge, considering that up to 80% of raw hides and skins treated to make leather are converted in solid wastes during the mechanical steps of the whole process (Kanagaraj et al., 2006; Onukak et al., 2017; Yorganicioglu et al., 2020).
To make leather production more environmentally sustainable, leather industry needs to look at a broader framework, not only moving towards cleaner production methods, but also improving its circular flow (Mu et al., 2003). In this perspective, leather industry needs to reduce, recover and reuse the amount of wastes (Li et al., 2019).
Solid wastes from pre-tanning and tanning processes, that amount to 99% of total solid wastes (Dixit et al., 2015; Kanagaraj et al., 2015), are composed by 30% of proteins, represented by 90-95% of collagen (Kite and Thomson, 2006). Several treatments of leather solid wastes have been developed to solve the environmental issue, allowing their conversion into highly added value products, such as re-tanning agents (Hu et al., 2011) and proteins (Taylor et al., 1996).
Although collagen recovery is, nowadays, a well-known process in the tanning industry, conventional procedures of collagen extraction from leather wastes are mainly based on chemical methods. Acid or alkaline agents are, in fact, used to recover collagen from the leather matrix (Ding et al., 2015; Taylor et al., 1996). However, the major

drawbacks of these methodology is the low quality of the recovered product (Qiang and Feng, 2011): through these procedures, indeed, the extracted collagen is hydrolyzed in small polypeptides (10-20 kDa), thus confining its applicability mainly to the agricultural field, as additive in the formulation of fertilizers (Nogueira et al., 2011).
Moreover, the recovery of already tanned wastes is complex, due to the high stability and complexity of the matrix and to the presence of polluting substances. Therefore, the tanned wastes require more extensively treatments that also integrate enzymatic hydrolysis to increase the extraction yields. However, the operative conditions of the extraction on tanned wastes result in a heterogeneous product that consists in high quality and extensively degraded collagen (Chen et al., 2001). On these types of wastes the main challenges of this industrial sector are currently focused.
As reported in the literature (Yorgancioglu et al., 2020), indeed, the enzymatic hydrolysis methods used still presents critical issues in terms of extraction yields, long reaction time, high costs and low quality of extracted collagen. The present work aims to develop innovative, economically viable, high effective, and environmental friendly protocols for the enzymatic extraction of collagen from leather tanned solid wastes.
The study is, therefore, focused on the development of enzymatic extraction methods that allow, when compared to those reported in literature (Sasia et al., 2019), to increase extraction yields and reduce reaction time. Moreover, quality of the extracted collagen has been assessed proving that these methods provide non-denatured collagen instead of partial degraded gelable polypeptides (Taylor et al., 1996).
The high quality of extracted collagen will affect its applicability: in particular, the extracted collagen could be applied *i)* as a replacement of conventional filling agents in the re-tanning process and *ii)* for the first time as the main component of a new finishing formulation. The integrity of extracted collagen is the key characteristic to exploit its efficiency and to confer to leather the desired functional features in terms of strength, fullness and softness. Moreover, to increase applicability of the extracted collagen, a cross-linking reaction with casein, catalyzed by a microbial transglutaminase, was set up and optimized.

Materials and Methods

Materials

Vegetable tanned bovine shavings (named S1), organic tanned bovine shavings (named S2) and mineral tanned bovine shavings (named S3) were collected from

the Italian Leather Research Institute (from the Veneto District), while chromium tanned bovine shavings (named S4) were collected from a local tannery (from the Campania District).

Sodium hydroxide, calcium hydroxide, casein from bovine milk, proteases (subtilisin A) 7 U/mg from *Bacillus licheniformis,* α-amylase 5 U/mg from porcine pancreas and trypsin 5-7 U/mg from porcine pancreas were purchased from Sigma-Aldrich (Milano, Italy).

Ammonium bicarbonate (AMBIC), acetonitrile, formic acid (HCOOH), sodium dodecyl sulphate (SDS), dithiothreitol (DTT), iodoacetamide (IAM) were purchased from Sigma-Aldrich (Milan, Italy). Sequential grade trypsin was from Promega (Milano, Italy).

Concentrated nitric (UpA) and hydrochloric (UpA) acids, and certified stock standard solutions were purchased from Romil Ltd (Cambridge, United Kingdom).

Microbial transglutaminase Activa WM was kindly furnished by Ajinomoto (Hamburg, Germany).

Collagen extraction

Enzymatic extraction methods for S1 and S2

1 g of wastes in 10 mL of distilled water was treated as follow:

- *no pre-treatment,* samples were incubated with 0.2% w/v of mixture of proteases, or 0.2% w/v mixture of amylase and proteases, or 0.2% w/v trypsin or no enzyme
- *physical pre-treatment*: 1 g of shavings was incubated at 100°C for 1h and then with 0.2% w/v of mixture of proteases, or 0.2% w/v mixture of amylase and proteases, or 0.2% w/v trypsin or no enzyme
- *chemical pre-treatment*: samples were incubated with 0.1, or 0.2, or 0.3% w/v of NaOH or $Ca(OH)_2$ for 1 h at RT and then with 0.2% w/v of mixture of proteases, or 0.2% w/v mixture of amylase and proteases, or 0.2% w/v trypsin or no enzyme

Once selected the sodium hydroxide for the pre-treatment and the trypsin for the enzymatic hydrolysis, the extraction was performed varying the concentration of NaOH (0.1, 0.2, 0.3 % w/v) and the incubation time of chemical pre-treatment (1, 2, 4 h).

Once selected the pre-treatment, the extraction was performed varying the concentration of the enzyme (0.1, 0.2, 0.4% w/v).

After enzymatic incubation, the enzyme was inactivated at 100°C for 5 min. The solution was filtered through Whatman No. 1 (Sigma Aldrich, Milano, Italy) filter paper under vacuum condition. The protein concentration was assayed though the Pierce 660 nm protein assay kit (Thermo Scientific, Waltham, MA) and through Bradford reagent (Bio-Rad protein assay). The BSA protein was used as standard protein for the calibration curve. Extracted collagen solutions were finally lyophilized through Savant ModulyoD Freeze Dryer of Thermo Electron Corporation.

Enzymatic extraction method for S3 and S4

1 g of wastes in 10 mL of distilled water were incubated at *i)* 100°C, 30 min; *ii)* ultrasonication, 10 min; *iii)* 100°C, 30 min and then ultrasonication, 10 min; *iv)* microwaves, 2 min; *v)* ultrasonication, 10 min and then microwaves, 2 min or *vi)* 120°C, 1 bar, 20 min. After the physical pre-treatment 0.5% w/v of NaOH were

added and stirred for 4 h at RT, then 0.2% w/v of trypsin was added and stirred for 2 h at 50°C.
Once selected the physical pre-treatment, it was performed for 5, 10 and 20 min.
Once selected the pre-treatment, the extraction was performed varying the concentration of the enzyme (0.1, 0.2, 0.4% w/v).
After the enzymatic incubation, the enzyme was inactivated at 100°C for 5 min and it was proceeded as described in section 2.2.1.

Chemical extraction method
Wastes were dried overnight through incubation into ventilated hoven at 60°C; 1 g of wastes was treated with 1.5% w/v of NaOH (Mu et al., 2003) in 10 mL of distilled water and stirred for 12h, 50°C. After incubation, it was proceeded as described in section 2.2.1.

Extraction yields
Extraction yields were calculated according to the protein content determined as reported in the section 2.2.1 on the weight of treated wastes (1), the protein content determined as reported in the section 2.2.1 on the total collagen content in treated wastes (2), where the total amount of collagen in 1 g of leather is assumed around 270 mg (Kite and Thomson, 2006), dry weight of extracts on dry weight of treated wastes (3) and dry weight of insoluble residue on dry weight of treated wastes (4).

$$Yield\ (\%) = \frac{protein\ content\ (mg)}{dry\ weight\ of\ treated\ wastes\ (mg)} \times 100 \qquad (1)$$

$$Yield\ (\%) = \frac{protein\ content\ (mg)}{total\ collagen\ content\ (mg)} \times 100 \qquad (2)$$

$$Yield\ (\%) = \frac{dry\ weight\ of\ extract\ (mg)}{dry\ weight\ of\ treated\ wastes\ (mg)} \times 100 \qquad (3)$$

$$Yield\ (\%) = \frac{dry\ weight\ of\ insoluble\ residue\ (mg)}{dry\ weight\ of\ treated\ wastes\ (mg)} \times 100 \qquad (4)$$

Characterization of extracted collagen
Extracted collagens were characterized in terms of molecular weights distribution, infrared analysis, protein identification, phenols content, formaldehyde and metal content.

Size exclusion chromatography
Samples of extracted collagen were suspended in 10 mM sodium phosphate buffer, pH 7.1, containing 150 mM NaCl (used also as elution buffer) and filtered through a 0.2 µm membrane filter. The size exclusion chromatography (SEC) was performed injecting 100 µL of sample at RT into a Superose 6 HR 10/300 column and using a ÄKTA pure 25 protein purification system. The flow rate was set at 0.5 mL/min, monitoring the UV-absorbance of the eluate at 280 nm.
Column calibration (Fig. A.1) was performed using thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa) and conalbumin (75 kDa) from Gel Filtration Calibration Kit HMW (all from Global Life Sciences Solutions, USA LLC). The calibration curve (Fig. A.2) was constructed plotting the partition coefficient (K_{av}) versus the logarithm of the molecular weight. The partition coefficient (K_{av}) was calculated as:

$$K_{av} = \frac{V_e - V_0}{V_c - V_0} \qquad (5)$$

where K_{av} is the partition coefficient, V_e is the elution volume, V_0 is the void volume and V_c is the geometric column volume.

The elution volumes of collagen samples were determined and the corresponding K_{av} were calculated, their molecular weights were thus inferred from the calibration curve.

ATR-IR analyses

ATR-IR spectral measurements were performed by Spectrum One ATR-IR Spectrometer on lyophilized samples. Samples were pressed on the ZnSe crystal and spectra were recorded with a resolution of 4 cm^{-1} at the range from 600 cm^{-1} to 4000 cm^{-1}.

Protein analysis

S-Trap digestion was performed according to the manufacturer's protocol. Samples were solubilized in 25 µL of lysis buffer (5 % SDS, 50 mM AMBIC), reduced with DTT (final concentration of 20 mM) and incubated for 10 min at 95 °C. After cooling the protein solutions up to room temperature, IAM (final concentration of 40 mM) was added to alkylate the cysteine residues and incubated in the dark for 30 min. Aqueous phosphoric acid (final concentration of 1.2 %) was added to stop the alkylation reaction.

Colloidal protein particulate was formed by adding 150 µl of S-Trap binding buffer (90% aqueous methanol, 100 mM AMBIC, pH 7.1) to the protein solutions. The mixture was placed on 2 mL micro S-Trap columns and centrifuged at 4000 rpm for 30 seconds. The columns were washed three times with 200 µL of S-Trap binding buffer and discarded after each wash step. Samples were subjected to enzymatic digestion by using a trypsin solution (0.12 µg/µL) at an enzyme-to-substrate ratio of 1:20 (w/w). The digestion was performed for 1 h at 47 °C. Peptides were eluted with 80 µL of 50 mM AMBIC followed by 80 µL of 0.2% aqueous formic acid, 40 µL of 50% acetonitrile and finally 40 µL of 70% acetonitrile both containing 0.2% HCOOH. The peptides were dried under vacuum and finally suspended in 50 µL of 0.1% HCOOH for a further desalting step using manually equipped tips with three Empore disc C18 (Merck, Darmstadt, Germany).

Peptide mixtures were analyzed by a 6520 Accurate-Mass Q-TOF LC/MS system (Agilent Technologies) equipped with a 1200 HPLC system and a chip cube (Agilent Technologies). After loading, the peptide mixtures (1 µL) were concentrated and desalted at flow rate of 4 µL/min in a 40 nL enrichment column with 0.1% HCOOH as eluent.

The samples were then fractionated on a C18 reverse phase capillary column (75 µm × 43 mm in the Agilent Technologies chip) at flow rate of 400 nL/min, with a linear gradient of eluent B (0.1% HCOOH in 95% ACN) in A (0.1% HCOOH in 2% ACN) from 5 to 80% in 50 min.

Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range m/z 300–2,400) followed by MS/MS scan of the five most abundant ions in each MS scan. MS/MS spectra were measured automatically when the MS signal was greater than the threshold of 50,000 counts. Double, triple and quadruple charge ions were preferably isolated and fragmented over singly charged ions. Data were acquired through Mass Hunter software (Agilent Technologies) and transformed in.mgf format and used for protein identification with a licensed version of Mascot Software1. Every protein was selected as significant when at least 2 peptides displayed a p value < 0.05.

Metal content

The metal content was determined through ICP-MS analysis. Standard solutions of

each metal were prepared in 3% nitric acid at five different concentrations (0, 1, 10, 50 and 100 µg/L). To mineralize the samples, concentrated nitric and hydrochloric acids were added to each samples (in the ratio of 1:3) and incubated for 16 hours at 90°C. The samples were then diluted in 50 mL of milli-Q water and transferred into a ICP-MS vial.

The metal concentrations measurement was performed with an Agilent 7700 ICP-MS instrument (Agilent Technologies) equipped with a frequency-matching radio frequency (RF) generator and 3rd generation Octapole Reaction System (ORS3), operating with helium gas in ORF. The following parameters were used: RF power 1550 W, plasma gas flow 14 L/min; carrier gas flow 0.99 L/min; He gas flow 4.5 mL/min. 103Rh was used as an internal standard (final concentration: 50 µg L−1).

To determine the content of chromium (VI) of S4, the samples were prepared according to the UNI EN ISO 4044:2017 and the analysis was performed according to the UNI EN ISO 17075-2:2017.

Formaldehyde content

To determine the content of formaldehyde of the extracted collagens, the samples were prepared according to the UNI EN ISO 4044:2017 and the analysis was performed according to the UNI EN ISO 17226-1:2020.

Costs analysis

The extraction collagen costs were calculated at laboratory scale, only considering the reactant, water and electricity costs, as reported in the supplementary materials.

Chemical cross-linking

Lyophilized samples of extracted collagen were suspended (10 mg/mL) in distilled water, 1% of glutaraldheyde was added and gently shaken. Once reaction was concluded, the resulted gels were heated at 70°C.

Enzymatic cross-linking

Collagen and casein were solubilized in 50 mM sodium phosphate buffer (1 mg/mL) at their suitable pH value at 6.0 and 8.0, respectively. Collagen and casein combined with different ratios (1:1, 1:0.2 and 1:0.1) were then incubated with transglutaminase at different concentrations (0, 25, 50, 100 U/g) and stirred for 2, 4 or 6 h at 40°C. After enzymatic incubation, the enzyme was inactivated by incubating the solutions at 100°C for 5 min, before to be lyophilized.

Characterization of cross-linked collagen

Cross-linked collagen was analyzed by SDS-PAGE and characterized in terms of molecular weights distribution and infrared analysis.

Electrophoresis in denaturing conditions, SDS-PAGE

Samples were treated with Sample Buffer and 10 mM DTT, then are loaded onto 10% polyacrylamide gel (10% w/v acrylamide, 0.1% w/v bisacrylamide, 0.4 M TrisHCl pH 9.2, 0.1% w/v APS, 0.001% TEMED). The electrophoretic run was performed in 0.1 M Tris-glycine pH 8.3 buffer with a current intensity of 25 mA. The gels were stained by silver staining.

Size exclusion chromatography

Samples of cross-linked collagen were suspended in 10 mM sodium phosphate buffer, pH 7.1, containing 150 mM NaCl (used also as elution buffer) and filtered

through a 0.2 µm membrane filter. The size exclusion chromatography (SEC) was performed as reported in the Section 2.3.1.

ATR-IR analyses
ATR-IR spectral measurements were performed as reported in the Section 2.3.2.

Statistics
All the experiments were performed at least in triplicate.

Results and discussion

Enzymatic extraction of collagen

Shavings tanned by different methods were treated by chemical, physical, and enzymatic methods or a combination of them in order to extract collagen. As reported in the literature (Godwin Jenifa et al., 2020; Mu et al., 2003; Sasia et al., 2019; Taylor et al., 1996; Yuanlong et al., 2012), the most efficient enzymatic treatment for collagen extraction from leather solid wastes consist of alkaline proteases and trypsin incubation. Unlike collagenases, these enzymes can act to break peptide bonds also on tanned collagen (Dettmer et al., 2013). In the present work, trypsin, a proteases mixture, and a proteases and amylases mixture were tested.
Preliminary experiments were performed on vegetable tanned bovine shavings (S1), where collagen extraction is expected to be easier compared to the other wastes due to the less strong interactions between tanning agents and collagen (mainly hydrogen bonds and electrostatic interactions).
Results shown in Figure 1 points out the necessity of a chemical pre-treatment, that facilitates the extraction of collagen by causing skins swelling (Schmidt et al., 2016). Chemical pre-treatment, moreover, allows reaching alkaline pH value (suitable for the activity of the tested enzymes). The combination of chemical pre-treatments with sodium hydroxide and trypsin resulted to be the most effective and promising approach among those tested.

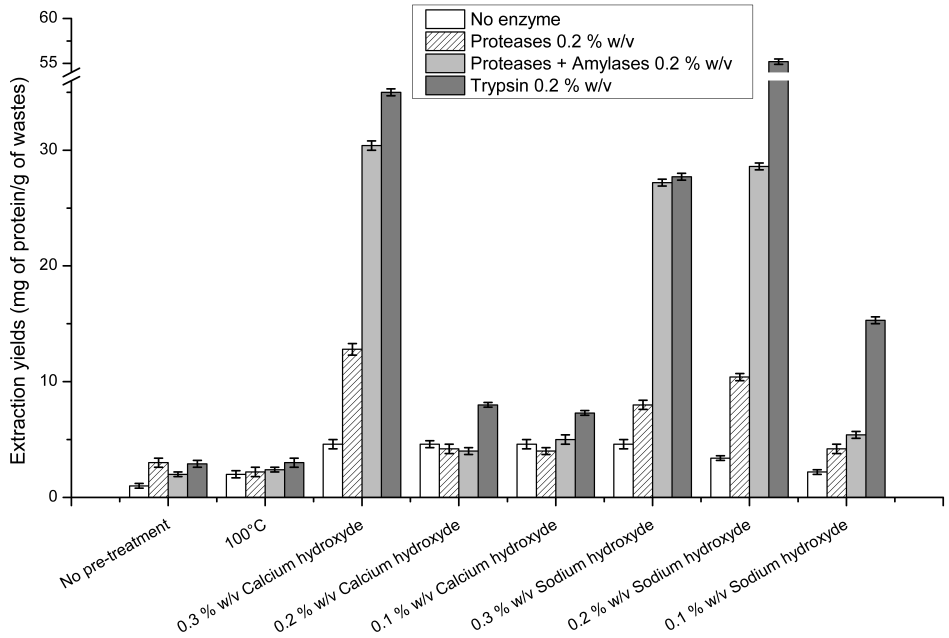

Figure 1. Extraction yields of collagen from S1

In order to increase extraction yields, pre-treatment time was extended (Fig. 2): the lowest concentration of sodium hydroxide integrated with trypsin allowed to reduce the amount of chemical used, as well as to use a cheaper chemical (sodium hydroxide instead of calcium hydroxide), obtaining higher amounts of extracted proteins. The condition that integrates the pre-treatment with 0.1 % w/v of NaOH for 4 h with the enzymatic treatment with 0.2 % w/v of trypsin for 2 h was selected.

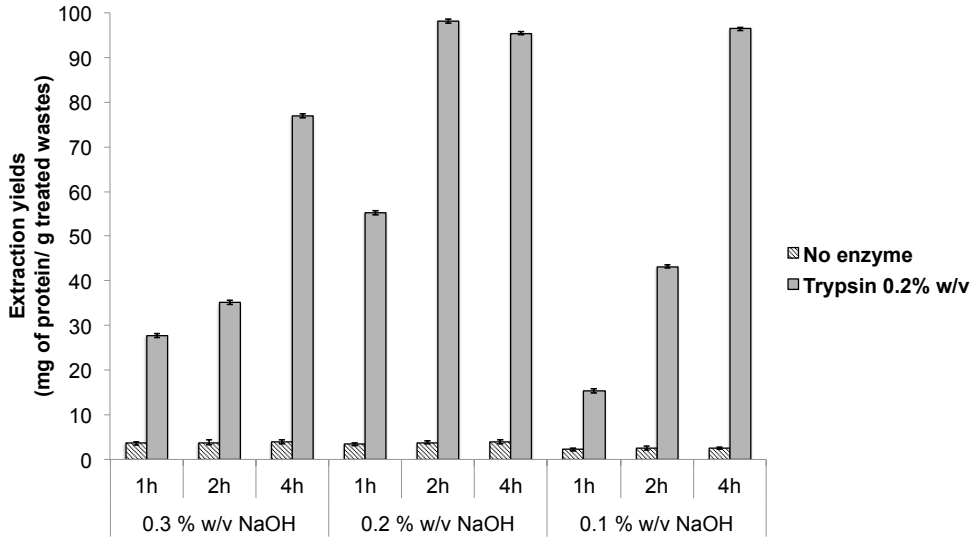

Figure 2. Extraction yields of collagen from S1 obtained by trypsin-mediated hydrolysis integrated with chemical pretreatment

As expected, the approach selected for S1 resulted to be effective for organic tanned bovine shavings (S2) too. Conversely, once the same protocol was applied on mineral tanned (S3) and chromium tanned (S4) bovine shavings, it resulted ineffective. The presence of more stable bonds between collagen fibers and the tanning agents results in a less degradable matrix. Therefore, sodium hydroxide concentration was raised up to 0.5% w/v and physical pre-treatments were introduced.

As reported in the literature (Scopel et al., 2019), physical pre-treatments, that provide sufficient energy to allow the breakage of chemical bonds between collagen and tanning agents, make collagen more accessible to the hydrolysis; thus, the only effective method to extract collagen from mineral tanned and chromium tanned bovine shavings resulted in a process that integrates high temperature and pressure exposition before chemical pre-treatment and enzymatic hydrolysis (Fig. 3).

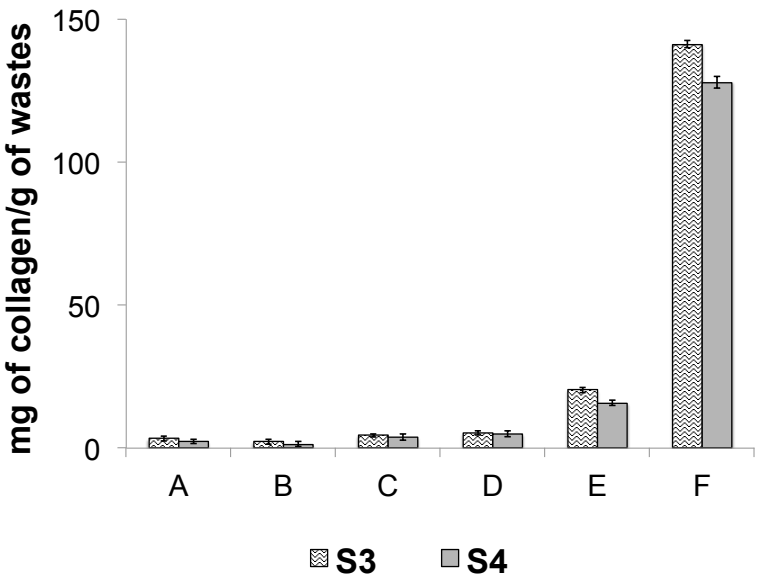

Figure 3. Extraction yields of collagen from S3 and S4 after chemical pre-treatment and enzymatic hydrolysis integrated with physical pretreatment: A) 100°C, 30 min; B) Ultrasonication, 10 min; C) 100°C, 30 min + ultrasonication, 10 min; D) Microwaves, 2 min; E) Ultrasonication 10 min + microwaves, 2 min; F) 120°C, 1 bar, 20 min.

The time of physical pre-treatment was decreased from 20 min to 5 min, reaching comparable extraction yields (140.4 ± 1.2 mg of protein/g of treated S3 and 129.3 ± 1.5 mg of protein/g of treated S4).
The integration of chemical and/or physical pre-treatment to enzymatic hydrolysis allowed to obtain higher extraction yields than those obtained by acid or alkaline treatments, reducing pollutant wastes and avoiding corrosive treatments to equipment (Gendaszewska et al., 2016; Rizk and Mostafa, 2016a).
Finally, to investigate the effect of enzymatic concentration, the extractions were performed following the selected methods and varying the enzymatic concentration (0.1, 0.2 or 0.4% w/v of trypsin). The amount of extracted collagen (Table A.1) resulted about 2% higher for the processes carried out with trypsin at 0.2 % w/v and 0.4 % w/v respect to the one carried out with 0,1% trypsin while the differences between the yields related to the processes performed with 0.2 % w/v and 0.4 % w/v of trypsin are negligible. Therefore, the 0.2 % w/v of enzyme was selected based on the cost-benefit criteria.

Collagen characterization

Protein identification analyses show that the proteins in the extracts belong to the collagen family; the α chains of various types of collagen, indeed, have been detected as follows:

- S1, bovine collagen type I (α2), type II (α1), type IV (α1) and type VIII (α2);
- S2, bovine collagen type I (α2), type II (α1), type IV (α1) and type VIII (α2);
- S3, bovine collagen type I (α1);
- S4, bovine collagen type I (α2), type II (α1), type IV (α1) and type VIII (α2).

Moreover, each sample presents different quantity of metals depending on the tanning method that the raw material has undergone. In particular, collagen from S3 is rich in aluminium probably due to the use of zeolite during the tanning process and collagen from S4 is rich in chromium due to its use as tanning agent (Table 1). Despite the high concentration in S4 of trivalent chromium (a not harmful substance, (Tegtmeyer and Kleban, 2014), it does not affect the applications of extracted collagen, i.e. the filling or finishing procedures during the post-tanning processes.

Finally, no toxic compounds were detected in all extracts: the determination of the content of hexavalent chromium in S4 (<3.0 mg/kg) proved that the developed extraction method do not cause the oxidation of trivalent chromium; and the content of formaldehyde in all extracts (<5.4 mg/kg) is lower of the limits imposed by UNI 10826:2012, UNI 10594:2010 and UNI 10885:2012.

Table 1. Metal content of the extracts from S1, S2, S3 and S4

	Metal content of extracted collagen			
	S1	**S2**	**S3**	**S4**
	ppm	ppm	ppm	Ppm
Beryllium	<0.001	<0.001	<0.001	<0.001
Aluminium	0.068	884.17	69494.95	2164.53
Silicon	0.315	1325.42	2314.40	1101.09
Vanadium	0.002	9.11	7.78	92.53
Chromium	0.117	152.35	387.80	113980.55
Manganese	0.002	26.16	12.35	15.23
Iron	0.451	853.67	330.39	582.94
Cobalt	<0.001	0.69	<0.001	1.34
Nickel	0.001	<0.001	<0.001	4.14
Copper	0.005	7.49	8.89	5.67
Zinc	0.085	7397.44	2566.76	3812.37
Selenium	<0.001	2.56	1.88	2.23
Molybdenum	<0.001	1.49	2.30	3.34
Cadmium	<0.001	<0.001	0.99	<0.001
Tin	<0.001	0.61	0.54	0.52
Antimony	<0.001	<0.001	<0.001	<0.001
Barium	0.006	16.53	133.32	17.26
Thallium	<0.001	<0.001	0.12	<0.001
Lead	<0.001	6.00	34.12	4.04

Comparison between enzymatic and chemical extraction methods

Two extraction methods were set up for metal-free tanned and metal tanned bovine shavings respectively, allowing to recover different types of collagen and, when compared to the chemical extraction methods (Fig. 4 and Table 2), to: *i*) increase the amount of extracted collagen (at least two times); *ii*) reduce the reaction time (up to two times); and *iii*) reduce the use of chemical agents (up to 10 times).

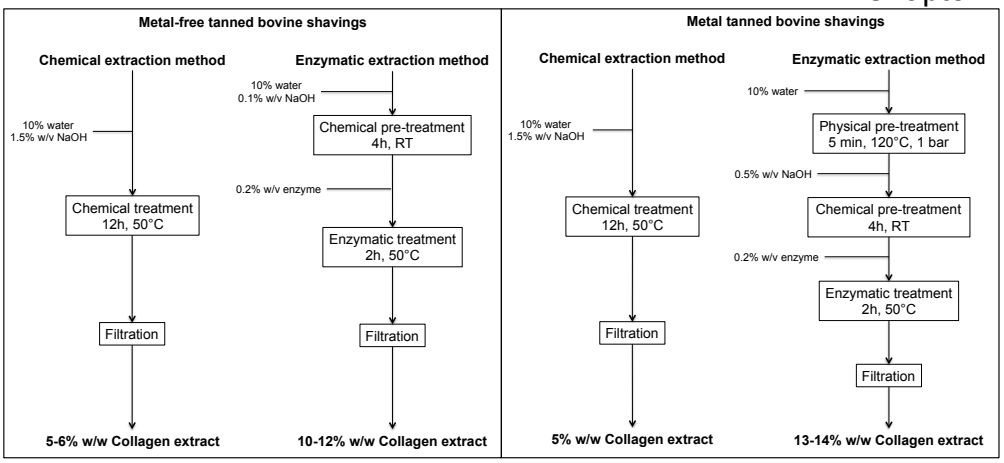

Figure 4. Outline of chemical and enzymatic procedures for the extraction of collagen from metal-free and metal tanned bovine shavings

Table 2. Collagen extraction yields (%w/w) from S1, S2, S3 and S4

	Extracted collagen/ Treated waste % (w/w)	Extracted collagen/ Total collagen* % (w/w)	Freeze-dried extract/ Treated waste % (w/w)	Insoluble residue/ Treated waste % (w/w)	Reaction time (h)	Chemicals used (%w/v)
S1	10.0±0.4[a]	37.0±0.5[a]	95.3±1.1[a]	2.3±0.2[a]	≈ 6[a]	0.1[a]
	5.1±0.3[b]	19±1.0[b]	78.2±0.8[b]	15±0.9[b]	≈ 12[b]	1.5[b]
S2	11.8±0.4[a]	43.7±1.0[a]	94.7±0.8[a]	2.5±0.5[a]	≈ 6[a]	0.1[a]
	6.2±0.2[b]	23±0.5[b]	73.0±1.2[b]	22±1.1[b]	≈ 12[b]	1.5[b]
S3	14.4±0.2[a]	53.3±0.5[a]	90.1±1.1[a]	7.1±0.6[a]	≈ 6.5[a]	0.5[a]
	4.9±0.5[b]	18±0.3[b]	71.2±1.0[b]	19.0±0.8[b]	≈ 12[b]	1.5[b]
S4	13.2±0.1[a]	48.9±0.3[a]	96.3±1.3[a]	2.0±0.1[a]	≈ 6.5[a]	0.5[a]
	5.2±0.3[b]	19±0.3[b]	59.0±1.0[b]	34.2±1.2[b]	≈ 12[b]	1.5[b]

*Calculated assuming that the total amount of collagen in 1 g of leather is around 270 mg (Kite and Thomson, 2006)
[a] Enzymatic extraction methods
[b] Chemical extraction method

Furthermore, the molecular weights (MWs) distribution of collagens extracted via enzymatic and chemical methods were analyzed and compared, suggesting that enzymatic extraction methods allow obtaining collagens of molecular weights higher compared to those of collagens obtained using the conventional chemical extraction method (Table 3, Fig. A.3-6).

Table 3. MWs distribution of collagen extracted via enzymatic and chemical methods from S1, S2, S3 and S4

Molecular weights distribution of extracted collagen

	Enzymatic method	Chemical method
S1	25-150 kDa	10-25 kDa
S2	20-70 kDa	10-20 kDa
S3	20-60 kDa	10-25 kDa
S4	40-150 kDa	10-35 kDa

The integrity of collagen extracted via enzymatic methods was also verified by ATR-IR: the stability of collagen can be analyzed through semi-quantitative relations between the signals detectable by IR analyses, which characterized the structural organization of collagen (Doyle et al., 1975; Oliveira et al., 2021; Rabotyagova et al., 2008).
In particular, the relations between the signals of the characteristic peaks of collagen are correlated to *i)* the maintenance of triple helix through the ratio between amide III and the absorbance at 1450 cm^{-1}, that correspond to the pyrrolidine rings of proline and hydroxyproline, (A_{III}/A_{1450}) (He et al., 2011; Silva et al., 2015; Sylvester et al., 1989; Vidal et al., 2020), which indicates the preservation of collagen conformation when its value is equal to unity; *ii)* the hydrolysis degree through the ratio between amide I and amide II (A_I/A_{II}) (Vyskočilová et al., 2019); and *iii)* the presence of denatured collagen when the difference between the frequencies of amide I and amide II (Δv) is major than 100 cm^{-1} (Albu et al., 2014; Ghica et al., 2009).
Comparing native and extracted collagens (Table 4), a common trend for all samples is highlighted: in native and enzymatic extracted collagens, the Δv displays the absence of denatured collagen, proving that enzymatic extraction methods resulted non-destructive. Moreover, the hydrolysis degrees (A_I/A_{II}) of enzymatic extracted collagens are comparable to those of native one, whereas collagens from chemical extraction method feature denatured collagen and high hydrolysis degrees, according to the results of molecular weights analysis. Additionally, while both native and enzymatic extracted collagens have high percentages of the preservation of their conformations (A_{III}/A_{1450}), in the spectra of chemical extracted collagens (Fig. A.7) the peak of amide III, which is correlated to triple helical structure and sensitive to protein structure alteration (Bet et al., 2001), is not detected, proving the loss of collagen conformation after the chemical hydrolysis.

Table 4. Semi-quantitative relations provided by ATR-IR analysis of native collagen, extracted collagen via enzymatic methods and chemical method of S1, S2, S3 and S4

		ATR-IR analyses		
		A_{III}/A_{1450}	A_I/A_{II}	Δv (cm^{-1})
S1	Native	0.98	0.98	79
	Via enzymatic method	0.99	0.98	81
	Via chemical method	Not detectable	1.5	102
S2	Native	0.99	0.87	82
	Via enzymatic method	0.97	0.94	83
	Via chemical method	Not detectable	1.6	137
S3	Native	0.99	0.90	84
	Via enzymatic method	0.99	0.91	93
	Via chemical method	Not detectable	1.3	100
S4	Native	0.98	0.91	79
	Via enzymatic method	0.99	0.92	95
	Via chemical method	Not detectable	2.5	167

Molecular weights distribution and infrared analyses highlighted the possibility to extract collagen without affecting its structure through enzymatic extraction methods, conversely to what results when using the chemical treatment. Therefore, not only the enzymatic extraction methods were proved to be applicable on all kinds of treated wastes, but also that these approaches are environmentally sustainable, *i.e.* reducing the use of polluting agents, and more effective in terms of the quality of extracted collagen.

Finally, a cost analysis of the collagen extraction processes was evaluated (Table A. 2-5): the costs related to the developed enzymatic methods were compared with the chemical one for each treated waste. The total costs of enzymatic extraction of collagen from each waste result slightly higher than the costs related to the chemical method. However, the productivity of the enzymatic methods is sensitively better that the chemical one, thus the cost of the former normalized by the amount of extracted collagen (expressed as euros per kilogram of extracted collagen) is about the half less expensive than the one of chemical process (Fig. 5).

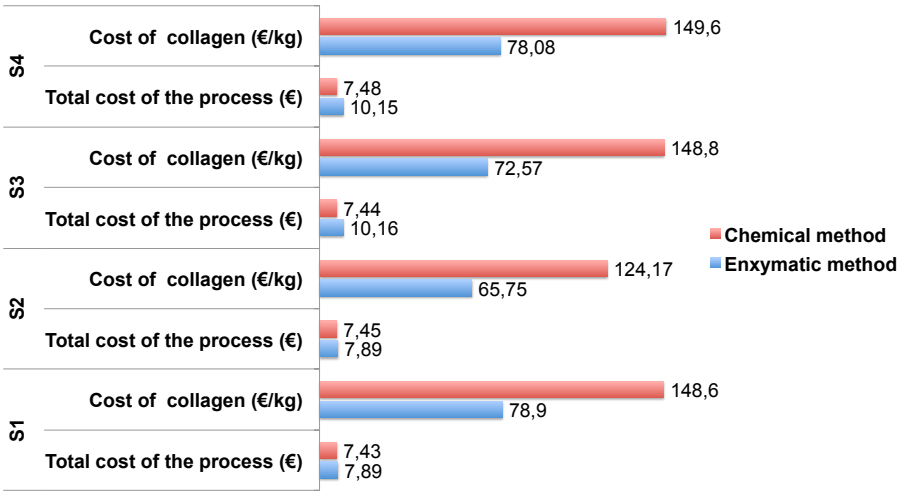

Figure 5. Total costs of chemical and enzymatic extraction processes and cost of extracted collagen from S1, S2, S3 and S4

Collagen cross-linking

Collagen cross-linking is an effective method to make collagen more suitable for several applications (Meena et al., 1999). Depending on the molecules involved, cross-linked collagen acquires new properties, such as thermal stability when cross-linked with casein (Taylor et al., 2006) or plastic properties when cross-linked to epichlorhydrin (Sundar et al., 2011).

Despite the presence of contaminants (section 3.2), the ability of cross-linking of the enzymatic extracted collagen, necessary to form intramolecular or intermolecular bonds between collagen and other molecules, was proved: once added the chemical cross-linker, the samples instantly gel at room temperature without liquefying at high temperatures (Fig. 6).

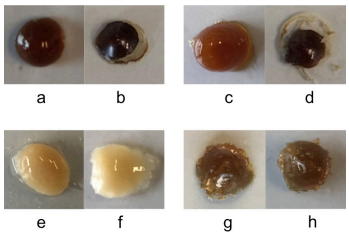

Figure 6. Cross-linked collagen before (a-c-e-g) and after (b-d-f-h) exposure at high temperature of collagen from S1 (a-b), S2 (c-d), S3 (e-f) and S4 (g-h)

The high quality of the extracted collagen and its ability to cross-link allowed the development of its enzymatic mediated cross-linking with casein. Cross-linking reaction was optimized varying: *i)* ratio between collagen and casein (1:1, 1:0.2, 1:0.1), *ii)* time of incubation (2h, 4h,

6h), *iii)* addition of casein (one-pulse and multi-pulse) and *iv)* enzymatic units (0, 25, 50, 100 U/g).

Time of incubation was assessed treating collagen and casein (1:0.2) with 50 U/g of transglutaminase for 2, 4, 6 h at the optimal temperature of the enzyme (40°C) and valuating the increase of molecular weights by SDS-PAGE (Taylor et al., 2006) (Fig. A.6). 2 h of incubation resulted to be not sufficient to obtain a significant increase of collagen molecular weights, while, the longest time of incubation (6 h) was not proportional to collagen molecular weights increasing, probably due to the long exposure of collagen to an high temperature that can cause its degradation (Ryhanen and Zaragoza, 1983). Conversely, carrying out the reaction for 4 h, higher molecular weights bands become more intense, while lower molecular weights bands tend to disappear.

The effect of the amount of enzyme was valuated incubating collagen and casein (1:0.2) with 0, 25, 50 and 100 U/g of transglutaminase (Fig. A.7). It resulted that at least 50 U/g of enzyme are necessary in order to obtain a noticeable increase of molecular weights.

The working conditions for collagen and casein cross-linking were optimized by analyzing the effect of the ratio between collagen and casein (1:1, 1:0.2 and 1:0.1) and the addition of casein (single-pulse or multi-pulse); and optimal conditions were selected on the basis of the percentage of high molecular weight moieties formed, by comparing chromatograms of treated and not treated collagen.

The molecular weights of collagen increased when treated with casein and enzyme (Table A.6 and Fig. A.8), this is a clear indication of cross-links formation between collagen and casein. Moreover, collagen-casein cross-linking is promoted and not casein-casein cross-linking not only by diminishing the amount of casein, but also by the multi-pulse addition of casein.

Furthermore, the formation of covalent bonds was determined as the conversion of free $–NH_2$ groups in –NH groups, thus the cross-linking degree was measured through the ratio between the amide I and amide A signals (A_I/A_A) of ATR-IR spectra (Albu et al., 2014; Garcia et al., 2007; Sionkowska et al., 2010; Sommer et al., 2021).

As reported in Table 5, cross-linking degrees, related to the products derived by all reaction conditions explored, are comparable except for the one related to the collagen and multi-pulse added casein (1:0.1) incubated with transglutaminase, which is higher than the others.

Table 5. Semi-quantitative relation provided by ATR-IR analysis of collagen, collagen incubated with transglutaminase, collagen and casein (1:0.1), collagen and

multi-pulse added casein (1:0.1) incubated with transglutaminase, casein incubated with transglutaminase

ATR-IR analyses

	A_I/A_A
Collagen	0.98
Collagen incubated with transglutaminase	0.99
Collagen and casein (1:0.1)	0.99
Collagen and casein (1:0.1) incubated with transglutaminase	1.4
Casein	1.0
Casein incubated with transglutaminase	1.0

Conclusions

The present work proposes new cost-effective collagen extraction methods that allow reducing the use of chemicals and reaction time and increasing extraction yields. The developed methods do not affect collagen integrity, allowing its applicability in filling and finishing post-tanning processes.

Finally, an enzymatic-mediated cross-linking between extracted collagen and casein was optimized *ex-situ*, allowing its exploitation during post-tanning processes.

CRediT authorship contribution statement

Marika Gargano: Conceptualization, Investigation, Methodology, Validation, Formal analysis, Visualization, Writing - original draft. **Claudia Florio**: Validation, Supervision, Writing - review & editing. **Angela Amoresano**: Validation and Supervision. **Giovanni Sannia, Vincenzo Lettera**: Conceptualization, Validation, Visualization, Supervision, Project administration, Writing - original draft, Writing - review & editing.

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Supplementary materials in press

Leather industry towards circular economy: enzymatic extraction of potential high added-value products from tanned wastes

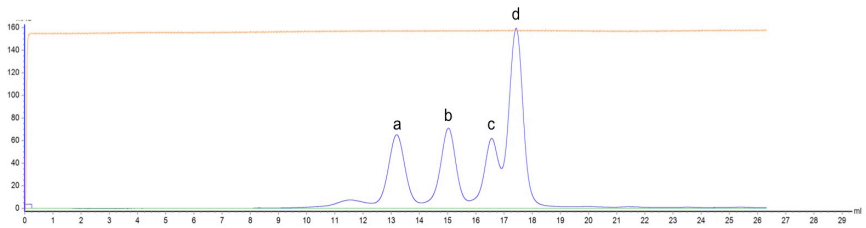

Figure A.1. Gel filtration chromatogram of a) thyroglobulin, b) ferritin, c) aldolase and d) conalbumin from Gel Filtration Calibration Kit HMW

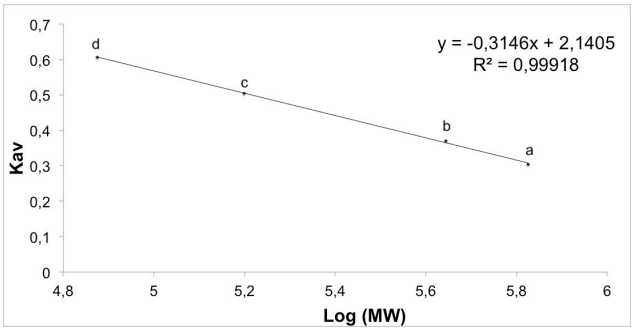

Figure A.2. Calibration curve of a) thyroglobulin, b) ferritin, c) aldolase and d) conalbumin from Gel Filtration Calibration Kit HMW

Table A.1. Collagen extraction yields from vegetable tanned bovine shavings (S1), organic tanned bovine shavings (S2), mineral tanned bovine shavings (S3) and chromium tanned bovine shavings (S4)

Extraction yields (mg of protein/ g of treated wastes)

	S1	S2	S3	S4
0.1 % w/v trypsin	72.2 ± 1.0	98.5 ± 1.1	119.5 ± 1.5	103.6 ± 1.2
0.2 % w/v trypsin	96.4 ± 0.1	118.3 ± 0.6	140.4 ± 1.2	129.3 ± 1.5
0.4 % w/v trypsin	101.3 ± 1.3	124.5 ± 1.1	143.2 ± 1.1	133.9 ± 1.3

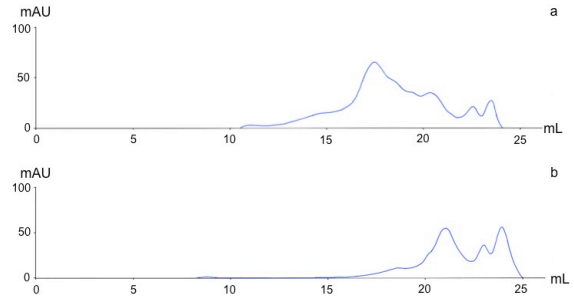

Figure A.3. Gel filtration chromatograms of collagen extracted from S1 via enzymatic method (a) and chemical method (b)

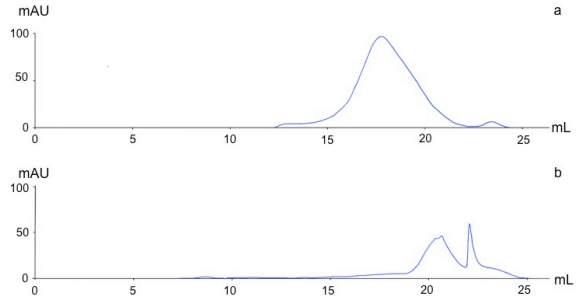

Figure A.4. Gel filtration chromatograms of collagen extracted from S2 via enzymatic method (a) and chemical method (b)

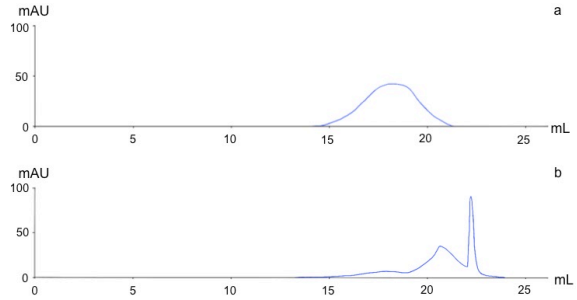

Figure A.5. Gel filtration chromatograms of collagen extracted from S3 via enzymatic method (a) and chemical method (b)

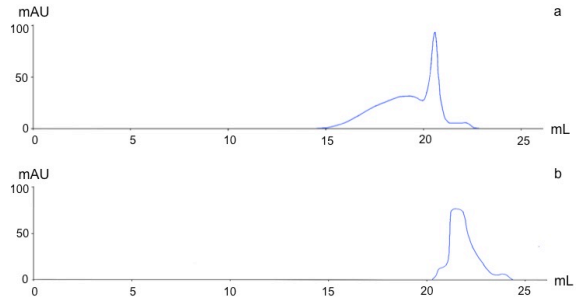

Figure A.6. Gel filtration chromatograms of collagen extracted from S4 via enzymatic method (a) and chemical method (b)

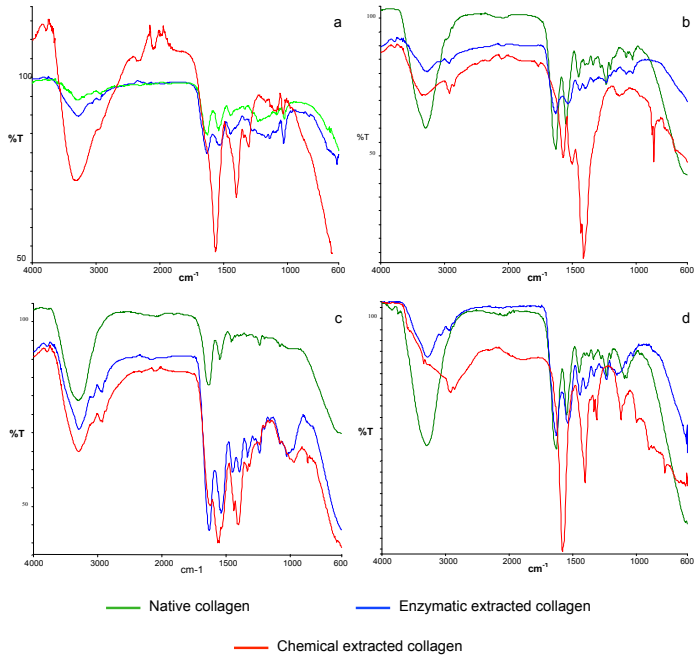

Figure A.7. ATR-IR spectra of S1 (a), S2 (b), S3 (c) and S4 (d)

Table A.2. Cost analysis for collagen extraction from S1 through chemical and enzymatic methods

	Costs related to the treatment of 1kg of S1							
	Chemical extraction				Enzymatic extraction			
	Input		Output		Input		Output	
	Amount	Cost	Amount	Cost	Amount	Cost	Amount	Cost
Process water [a]	0.01 m^3	€ 0.01	-	-	0.01 m^3	€ 0.01	-	-
Chemical treatment								
- NaOH [b]	0.15 kg	€ 3.11	-	-	0.01 kg	€ 0.21	-	-
- Temperature [c]	50°C	€ 4.11	-	-	RT	-	-	-
- Stirring [d]	150 rpm	€ 0.16	-	-	150 rpm	€ 0.05	-	-
- Process time	12 h	-	-	-	4 h	-	-	-
Enzymatic treatment								
- Enzyme [e]	-	-	-	-	0.02 kg	€ 6.89	-	-
- Temperature [c]	-	-	-	-	50°C	€ 0.69	-	-
- Stirring [d]	-	-	-	-	150 rpm	€ 0.03	-	-
- Process time	-	-	-	-	2 h	-	-	-
Extracted collagen	-	-	0.05 kg	-	-	-	0.10 kg	-
Insoluble residue [f]	-	-	0.15 kg	€ 0.04	-	-	0.02 kg	€ 0.01
Total costs	€ 7.43				€ 7.89			
Collagen extraction €·kg^{-1} [g]	€ 148.60				€ 78.90			

a) Water costs: 0.88 €·m^{-3} (Resolution ARERA n.665/2017/R/Idr)
b) NaOH costs: 20.72 €·kg^{-1} (the cheapest price present on the market related to the product with analogue characteristics)
c) Estimated as 1.27 kW per 0.27 €·kW^{-1}·h^{-1} per process time
Where 1.27 kW is the power required by the used oven (Heraus Function Line UT 6); 0.27 €·kW^{-1}·h^{-1} is the current cost of the electricity (Resolution ARERA n.621/2021/R/eel)
d) Estimated as 0.05 kW per 0.27 €·kW^{-1}·h^{-1} per process time
Where 0.05 kW is the power required by the orbital shaker (Stuart Scientific SSL1); 0.27 €·kW^{-1}·h^{-1} is the cost of electricity (Resolution ARERA n.621/2021/R/eel)
e) Trypsin costs is 344.40 €·kg^{-1} (the cheapest price present on the market related to the product with analogue characteristics)
f) Estimated as amount of residue per 0.28 €·kg^{-1}
Where 0.28 €·kg^{-1} is the cost related to tanned wastes disposing (Law decree BUR n.104-03122013)
g) Calculated as Total costs per kg of extracted collagen

Table A.3. Cost analysis for collagen extraction from S2 through chemical and enzymatic methods

	Costs related to the treatment of 1kg of S2							
	Chemical extraction				Enzymatic extraction			
	Input		Output		Input		Output	
	Amount	Cost	Amount	Cost	Amount	Cost	Amount	Cost
Process water [a]	0.01 m^3	€ 0.01	-	-	0.01 m^3	€ 0.01	-	-
Chemical treatment								
- NaOH [b]	0.15 kg	€ 3.11	-	-	0.01 kg	€ 0.21	-	-
- Temperature [c]	50°C	€ 4.11	-	-	RT	-	-	-
- Stirring [d]	150 rpm	€ 0.16	-	-	150 rpm	€ 0.05	-	-
- Process time	12 h	-	-	-	4 h	-	-	-
Enzymatic treatment								
- Enzyme [e]	-	-	-	-	0.02 kg	€ 6.89	-	-
- Temperature [c]	-	-	-	-	50°C	€ 0.69	-	-
- Stirring [d]	-	-	-	-	150 rpm	€ 0.03	-	-
- Process time	-	-	-	-	2 h	-	-	-
Extracted collagen	-	-	0.06 kg	-	-	-	0.12 kg	-
Insoluble residue [f]	-	-	0.22 kg	€ 0.06	-	-	0.02 kg	€ 0.01
Total costs	€ 7.45				€ 7.89			
Collagen extraction €·kg^{-1} [g]	€ 124.17				€ 65.75			

a) Water costs: 0.88 €·m^{-3} (Resolution ARERA n.665/2017/R/Idr)
b) NaOH costs: 20.72 €·kg^{-1} (the cheapest price present on the market related to the product with analogue characteristics)
c) Estimated as 1.27 kW per 0.27 €·kW^{-1}·h^{-1} per process time
Where 1.27 kW is the power required by the used oven (Heraus Function Line UT 6); 0.27 €·kW^{-1}·h^{-1} is the current cost of the electricity (Resolution ARERA n.621/2021/R/eel)
d) Estimated as 0.05 kW per 0.27 €·kW^{-1}·h^{-1} per process time
Where 0.05 kW is the power required by the orbital shaker (Stuart Scientific SSL1); 0.27 €·kW^{-1}·h^{-1} is the cost of electricity (Resolution ARERA n.621/2021/R/eel)
e) Trypsin costs is 344.40 €·kg^{-1} (the cheapest price present on the market related to the product with analogue characteristics)
f) Estimated as amount of residue per 0.28 €·kg^{-1}
Where 0.28 €·kg^{-1} is the cost related to tanned wastes disposing (Law decree BUR n.104-03122013)
g) Calculated as Total costs per kg of extracted collagen

Table A.4. Cost analysis for collagen extraction from S3 through chemical and enzymatic methods

	Costs related to the treatment of 1kg of S3							
	Chemical extraction				Enzymatic extraction			
	Input		Output		Input		Output	
	Amount	Cost	Amount	Cost	Amount	Cost	Amount	Cost
Water [a]	0.01 m^3	€ 0.01	-	-	0.01 m^3	€ 0.01	-	-
Physical treatment - 120°C, 1 bar [b]	-	-	-	-	5 min	€ 0.30	-	-
Chemical treatment								
- NaOH [c]	0.15 kg	€ 3.11	-	-	0.05 kg	€ 1.03	-	-
- Temperature [d]	50°C	€ 4.11	-	-	RT	-	-	-
- Stirring [e]	150 rpm	€ 0.16	-	-	150 rpm	€ 1,19	-	-
- Process time	12 h	-	-	-	4 h	-	-	-
Enzymatic treatment								
- Enzyme [f]	-	-	-	-	0.02 kg	€ 6.89	-	-
- Temperature [d]	-	-	-	-	50°C	€ 0.69	-	-
- Stirring [e]	-	-	-	-	150 rpm	€ 0.03	-	-
- Process time	-	-	-	-	2 h	-	-	-
Extracted collagen	-	-	0.05 kg	-	-	-	0.14 kg	-
Insoluble residue [g]	-	-	0.19 kg	€ 0.05	-	-	0.07 kg	€ 0.02
Total	€ 7.44				€ 10.16			
Collagen extraction €·kg^{-1} [h]	€ 148.80				€ 72.57			

a) Water costs: 0.88 €·m^{-3} (Resolution ARERA n.665/2017/R/Idr)
b) Estimated as 2.2 kW per 0.27 €·kW^{-1}·h^{-1} per 0.5 h
Where 2.2 kW is the power consumption of the used autoclave (Vapor Matic 770); 0.27 €·kW^{-1}·h^{-1} is the cost of electricity (Resolution ARERA n.621/2021/R/eel); and 0.5 h is the time that the autoclave takes to hold 120°C and 1 bar for 5 minutes
c) NaOH costs: 20.72 €·kg^{-1} (the cheapest price present on the market related to the product with analogue characteristics)
d) Estimated as 1.27 kW per 0.27 €·kW^{-1}·h^{-1} per process time
Where 1.27 kW is the power required by the used oven (Heraus Function Line UT 6); 0.27 €·kW^{-1}·h^{-1} is the current cost of the electricity (Resolution ARERA n.621/2021/R/eel)
e) Estimated as 0.05 kW per 0.27 €·kW^{-1}·h^{-1} per time
Where 0.05 kW is is the power required by the the orbital shaker (Stuart Scientific SSL1); 0.27 €·kW^{-1}·h^{-1} is the cost of electricity (Resolution ARERA n.621/2021/R/eel)
f) Trypsin costs is 344.40 €·kg^{-1} (the cheapest price present on the market related to the product with analogue characteristics)
g) Estimated as amount of residue per 0.28 €·kg^{-1}
Where 0.28 €·kg^{-1} is the cost related to tanned wastes disposing (Law decree BUR n.104-03122013)
h) Calculated as Total costs per kg of extracted collagen

Table A.5. Cost analysis for collagen extraction from S4 through chemical and enzymatic methods

	Costs related to the treatment of 1kg of S4							
	Chemical extraction				Enzymatic extraction			
	Input		Output		Input		Output	
	Amount	Cost	Amount	Cost	Amount	Cost	Amount	Cost
Water [a]	0.01 m^3	€ 0.01	-	-	0.01 m^3	€ 0.01	-	-
Physical treatment - 120°C, 1 bar [b]	-	-	-	-	5 min	€ 0.30	-	-
Chemical treatment								
- NaOH [c]	0.15 kg	€ 3.11	-	-	0.05 kg	€ 1.03	-	-
- Temperature [d]	50°C	€ 4.11	-	-	RT	-	-	-
- Stirring [e]	150 rpm	€ 0.16	-	-	150 rpm	€ 1,19	-	-
- Process time	12 h	-	-	-	4 h	-	-	-
Enzymatic treatment								
- Enzyme [f]	-	-	-	-	0.02 kg	€ 6.89	-	-
- Temperature [d]	-	-	-	-	50°C	€ 0.69	-	-
- Stirring [e]	-	-	-	-	150 rpm	€ 0.03	-	-
- Process time	-	-	-	-	2 h	-	-	-
Extracted collagen	-	-	0.05 kg	-	-	-	0.13 kg	-
Insoluble residue [g]	-	-	0.34 kg	€ 0.09	-	-	0.02 kg	€ 0.01
Total	€ 7.48				€ 10.15			
Collagen extraction €·kg^{-1} [h]	€ 149.60				€ 78.08			

a) Water costs: 0.88 €·m^{-3} (Resolution ARERA n.665/2017/R/Idr)
b) Estimated as 2.2 kW per 0.27 €·kW^{-1}·h^{-1} per 0.5 h
Where 2.2 kW is the power consumption of the used autoclave (Vapor Matic 770); 0.27 €·kW^{-1}·h^{-1} is the cost of electricity (Resolution ARERA n.621/2021/R/eel); and 0.5 h is the time that the autoclave takes to hold 120°C and 1 bar for 5 minutes
c) NaOH costs: 20.72 €·kg^{-1} (the cheapest price present on the market related to the product with analogue characteristics)
d) Estimated as 1.27 kW per 0.27 €·kW^{-1}·h^{-1} per process time
Where 1.27 kW is the power required by the used oven (Heraus Function Line UT 6); 0.27 €·kW^{-1}·h^{-1} is the current cost of the electricity (Resolution ARERA n.621/2021/R/eel)
e) Estimated as 0.05 kW per 0.27 €·kW^{-1}·h^{-1} per time
Where 0.05 kW is is the power required by the the orbital shaker (Stuart Scientific SSL1); 0.27 €·kW^{-1}·h^{-1} is the cost of electricity (Resolution ARERA n.621/2021/R/eel)
f) Trypsin costs is 344.40 €·kg^{-1} (the cheapest price present on the market related to the product with analogue characteristics)
g) Estimated as amount of residue per 0.28 €·kg^{-1}
Where 0.28 €·kg^{-1} is the cost related to tanned wastes disposing (Law decree BUR n.104-03122013)
h) Calculated as Total costs per kg of extracted collagen

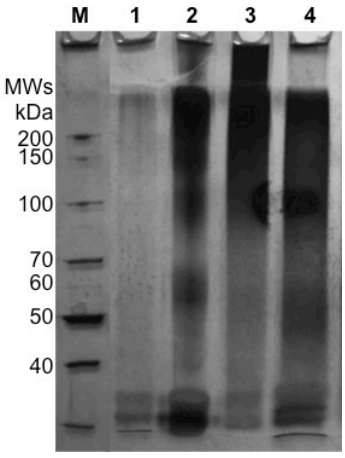

Figure A.6. Gel SDS-PAGE of M) protein standard, 1) casein, 2) collagen and casein 1:0.2 incubated with 50 U/g of transglutaminase for 6h, 3) collagen and casein 1:0.2 incubated with 50 U/g of transglutaminase for 4h and 4) collagen and casein 1:0.2 incubated with 50 U/g of transglutaminase for 2h

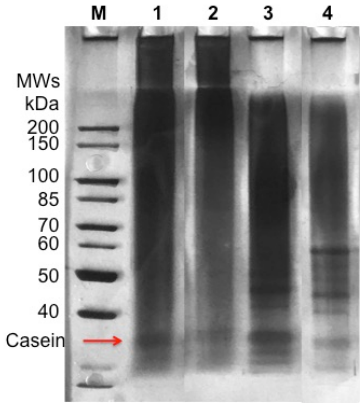

Figure A.7. Gel SDS-PAGE of M) protein standard, 1) collagen and casein 1:0.2 incubated with 100 U/g of transglutaminase for 4h, 2) collagen and casein 1:0.2 incubated with 50 U/g of transglutaminase for 4h, 3) collagen and casein 1:0.2 incubated with 25 U/g of transglutaminase for 4h and 4) collagen and casein 1:0.2 incubated with 0 U/g of transglutaminase for 4h

CHAPTER 2 - VALORIZATION OF LEATHER SOLID WASTES: APPLICATION OF EXTRACTED COLLAGEN

Post-tanning process consists in a series of operations that aim to improve the aesthetic properties of leather. This last step of leather processing produces low amount of solid wastes and wastewaters rich in polluting and potentially toxic chemicals. Leather industry is, therefore, redesigning post-tanning methods by the replacement of toxics with bio-based compounds and by the introduction of enzymatic processes, without sacrificing the quality of the product (Ollé et al., 2009).

In this scenario, using of collagen recovered from leather wastes can not only replace chemicals, assuring high compatibility with leather, but also drive the transition to circularity, converting wastes into valuable products.

In this chapter, collagen extracted from tanned solid wastes has been applied both as filling agent during re-tanning process through an *in situ* enzymatic cross-linking and as finishing agent, replacing non-biodegradable resins.

References

Ollé, L., Cobos, M., Solé, O., and Bacardit, A. (2009). Aqueous finishing with polyisocyanate cross-linked binders. *J. Soc. Leather Technol. Chem.* 93, 222–228.

Clean Technologies and Environmental Policy
Focusing on Technology Research, Innovation, Demonstration, Insights and Policy Issues for Sustainable Technologies

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From leather wastes to leather: enhancement of low quality leather using collagen recovered from leather tanned wastes

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Abstract

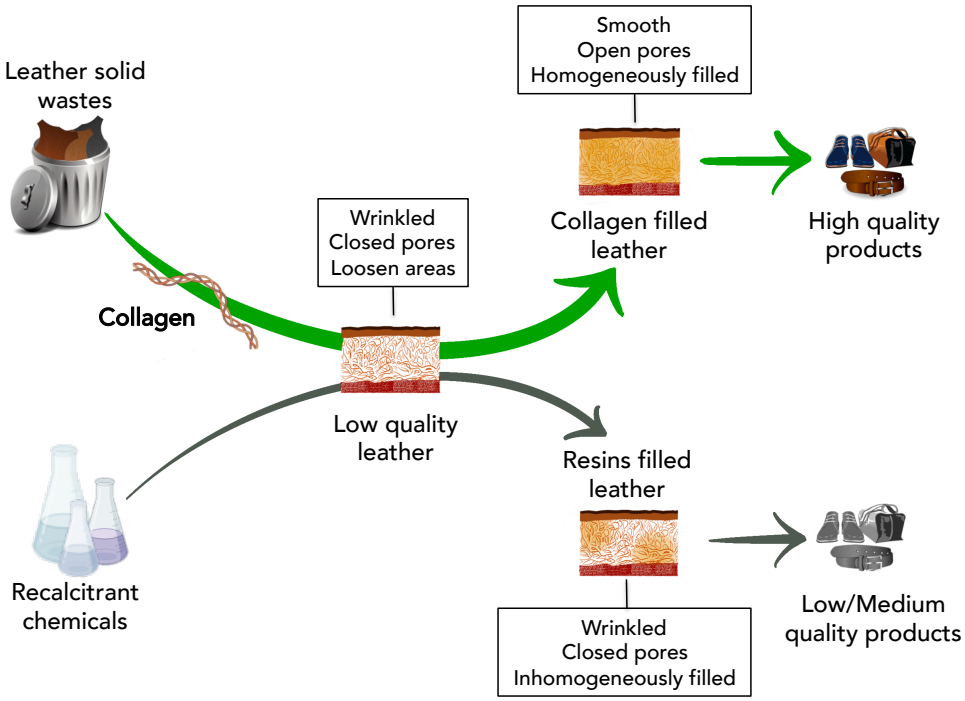
Leather industry produces huge amounts of solid wastes. In the last decade, several methods for the recovery and valorization of these wastes were developed, mainly focused on the extraction of collagen using chemical methods. The extracted collagen, due to its poor quality, is mostly used in agriculture as a nitrogen source ingredient of fertilizers. This study aims to apply collagen, extracted from leather tanned solid wastes using a recently reported new process based on enzymatic hydrolysis, as filling agent for low quality leather. Thanks to the enzymatic hydrolysis, collagen can be extracted without affecting its integrity and, therefore, its quality.

In order to use the extracted collagen as filler for low quality leather, an enzymatic mediated cross-linking reaction between collagen and casein was developed. The enzymatic cross-linking reaction was added as an additional phase of the re-tanning process or as a replacement of one of the re-tanning steps. To evaluate the filling effect, thickness of the treated leather was measured and infrared and microscopy analyses were performed, comparing the new methods to the traditional standard one. The mechanical properties of the filled leather were tested and the sensorial features, such as fullness and touch feelings, were estimated through a panel test. Results suggest

the high potential of extracted collagen to be employed back in leather processing both as additive and as substitutive filler.

Keywords: Waste re-use; clean process; enzymatic cross-linking; circular economy; closed-loop

Graphical abstract



Introduction

Leather making process is long and complex: animal hides and skins derived from meat industry are undergone to a series of operations to be converted in leather (Maina et al. 2019). Leather processing is commonly divided in three main phases: pre-tanning, tanning and post-tanning. The pre-tanning process comprises activities to clean and store hides; the tanning process converts hides in leather, by modifying the collagen present in the skin fibers and the post-tanning steps are responsible of the final aspect and of the aesthetic and mechanical properties of leather (Thanikaivelan et al. 2005).

Among post-tanning procedures, the re-tanning process is crucial not only to achieve uniform leather products, but also to confer new properties to leather, thus increasing its quality (Yorganicioglu et al. 2020): frequently, the addition of fillers is necessary to minimize the imperfections present on hides, such as veiny and loose areas (Taylor

et al. 2009). Filling phase is, therefore, the introduction of substances able to penetrate leather and fill the voids of the fibers (Taylor et al. 2006). The formulation of the filling agents determines the final properties of the treated leather (Danylkovych and Korotych 2019): the ideal filler has to be highly compatible with leather, well soluble in water, capable to penetrate into leather matrix and to bond itself to collagen, and able to homogeneously fill the loosen parts (Yorgancioglu et al. 2020).

To achieve these desired characteristics, several inorganic or organic chemicals are generally used simultaneously; among the organic materials, four classes of compounds are the most used in re-tanning process: i) vegetal tannins, ii) syntans, iii) resins and iv) polymers (Jankauskaite et al. 2012).

In the last decade, the high production costs and the low degree of penetration of vegetal tannins in leather (Mokrousova 2010; Jankauskaite et al. 2012), as well as the potential release of polluting and harmful substances (such as free-formaldehyde) due to the use of synthetic fillers (Yorgancioglu et al. 2020), have promoted the development and the application of alternative eco-friendly and non toxic compounds, such as proteins and oligopeptides.

Among proteins, collagen resulted to be a very interesting alternative filler (Chen et al. 2001) thanks to its structural function and its compatibility with leather (Sun et al. 2022). In particular, by its cross-linking combined with other molecules, collagen attains new appealing properties, such as thermal stability, elasticity and plasticity (Zehra et al. 2019; Skopinska-Wisniewska et al. 2021). In particular, collagen-casein cross-linking is the key for collagen application in leather processing, being casein able to make collagen more resistant to the thermal stress caused by the re-tanning process (Wu et al. 2017).

Potential collagen sources are numerous, from mammals to marine organisms (Silvipriya et al. 2015), including tannery wastes: leather solid wastes are, indeed, composed by raw hides and by semi-processed skins, where collagen is the main protein component (Kite and Thomson 2006).

Aiming to a cleaner and circular production flow, applying extracted collagen from leather solid wastes as filler into leather processing has been more than a challenge. Employing back collagen in leather manufacturing has also the advantage of recovering a waste without the need of further purification steps, steps which are necessary for the reuse of collagen in other fields, such as cosmetic and medical applications (Sionkowska et al. 2017).

Moreover, leather solid wastes represent a critical issue for the environmental sustainability of the leather processing industry: tanned shavings result to be high polluting and potentially toxic due to the presence of tanning agents that, bonding to collagen fibers in the skin, make these wastes hard degradable.
Nowadays, several methods, based on chemical extraction for collagen recovery, were developed (Xiaoyan et al.; Ding et al. 2015), but the obtained collagen is completely deconstructed and hydrolyzed in small polypeptides (Nogueira et al. 2011), making it hard to be applied in the filling process.
In our previous work, collagen was extracted from leather tanned wastes by enzymatic hydrolysis, thus allowing to recover high quality structured collagen, and providing a protocol for its cross-linking with casein by enzymatic catalysis. Using of enzymes allow to fine tuning the hydrolysis and cross-linking processes, making extracted collagens suitable for application in leather manufacturing.
In the present study, collagen, extracted from different kind of tanned shavings, is integrated in leather manufacturing as filler for low quality leather, exploiting its ability to cross-link with casein through the mediation of a microbial transglutaminase. The re-tanning process was redesigned to apply collagen as filler additive or substitute.

Materials and methods

Materials

Vegetable tanned bovine shavings, organic tanned bovine shavings and mineral tanned bovine shavings were collected from the Italian Leather Research Institute (form Veneto district).
Ovine leather and re-tanning reagents were furnished by DMD Solofra Spa - Tanning Company.
Reagents, casein and trypsin were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). Microbial transglutaminase Activa WM was furnished by Ajinomoto (Hamburg, Germany).

Extraction of collagen

Vegetable tanned bovine shavings, organic tanned bovine shavings and mineral tanned bovine shavings were treated with enzymatic treatment to extract collagen as reported in our previous work.

Application of collagen as filler

Collagen with molecular weights in the range of 25-150 kDa (5% w/w for each gram of treated leather), casein (0.5% w/w for each gram of treated leather) and transglutaminase (50 U/g of collagen) were applied as additive or as substitutive filler as reported in figure 1 during different steps of re-tanning process.

Scanning electron microscopy

Test samples and control were cut in small pieces. A sputter coater (Dynavac) was used for coating a layer of gold on the surface of all samples that were fixed on aluminum stub.
A Zeiss EVO MA10 Scanning Electron Microscope equipped with INCA X-act ENERGY-250XT detector was used for the analyses (magnification 10x - 200000x, resolving power 2 μm).

Stereomicroscopy
Test samples and control were set in distilled water overnight and then freezed to cut thin sections by a micrometer. Each section was analyzed by Wild Heerbrugg Stereoscope equipped with OPTIKA Room B3 Digital Camera.

Filling efficiency
Leather thickness was measured before and after re-tanning treatment by a Thickness Tester IG/MS, according to the standard EN ISO 2589:2016. Filling efficiency was calculated using the following formula (Chen et al. 2001):

$$\Delta T(\%) = \frac{T_a - T_b}{T_b} \times 100$$

where T_a is the thickness after the treatment and T_b is the thickness before the treatment.

ATR-IR
ATR-IR (Attenuated Total Reflection – InfraRed) spectral measurements were performed by Spectrum One ATR-IR Spectrometer on test samples and control. Samples were pressed on the ZnSe crystal and spectra were recorded with a resolution of 4 cm^{-1} at the range from 600 cm^{-1} to 4000 cm^{-1}.

Mechanical tests of treated leather
Mechanical properties of test samples and control were evaluated as follow: i) shrinkage temperature according to UNI EN ISO 3380:2015; ii) tensile strength and percentage of elongation according to UNI EN ISO 3376:2016; iii) tear strength according to UNI EN ISO 3377-2:2016; and iv) distention and strength of surface according to UNI EN ISO 3379:2015 (ball burst method).

Results and discussion

Application of extracted collagen and casein as filling agents

In our previous study, the enzymatic cross-linking reaction between collagen and casein was optimized *ex situ,* in terms of time of reaction, enzymatic units used and collagen-casein ratio. To apply the extracted collagen and casein as filling agents, the enzymatic cross-linking was carried out *in situ* in the conditions optimized for the *ex situ* reaction.
The re-tanning process was re-designed to spread extracted collagen and casein as fillers additive or substitute on low quality leather (characterized by the presence of holes, wrinkles and inhomogeneity of the surface) through the mediation of transglutaminase (Fig. 1).

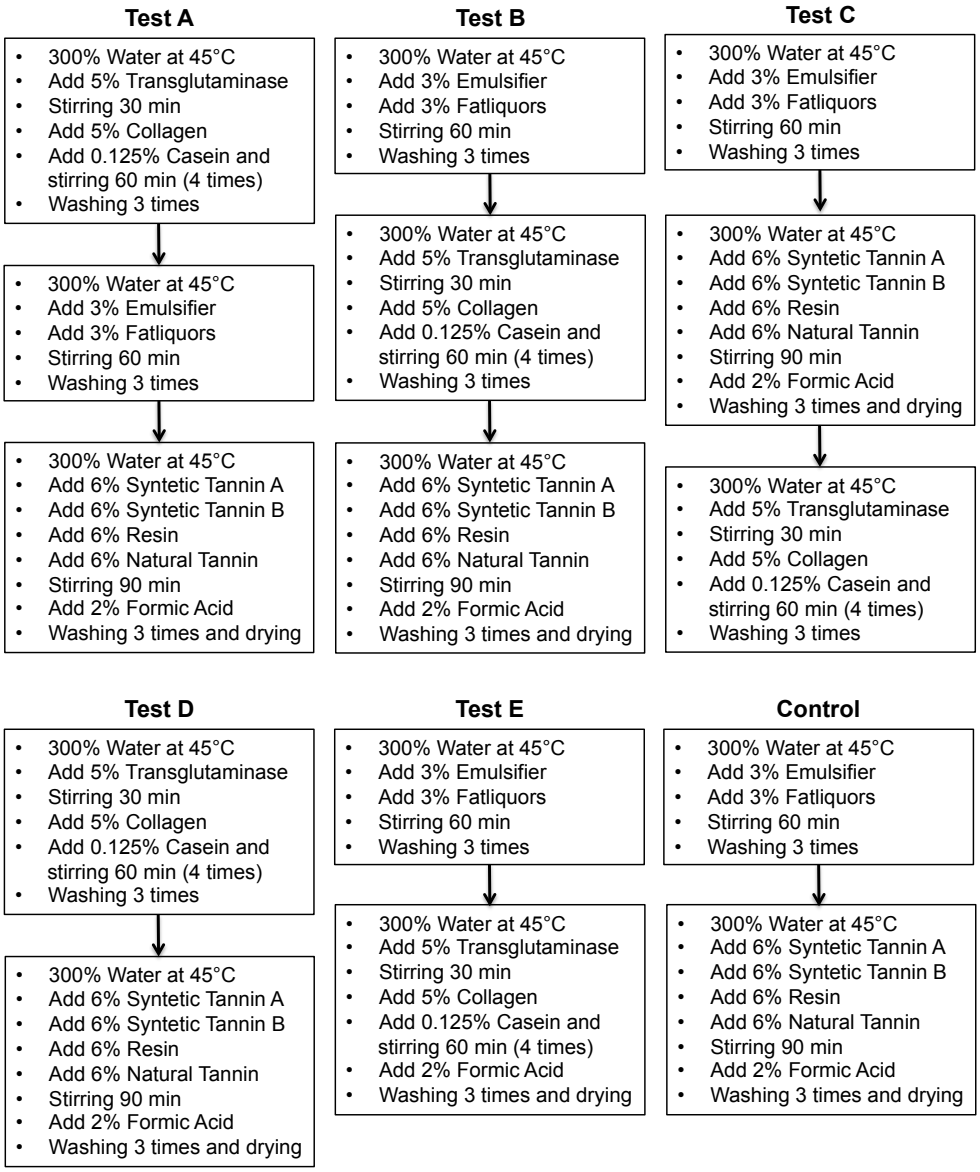


Fig. 1 Redesigned re-tanning protocols for the application of extracted collagen as additive (Test A, B and C) or substitute (Test D and E) filler

The thickness of all samples was measured before and after the treatments and the related filling efficiencies were calculated (Table 1): thickness increases after the treatments in all samples but Test D, as a consequence of the filling agents penetration in the leather matrix.

In particular, Tests A, B and E have a comparable filling efficiency, doubled respect to the control, showing that not only the addition of

collagen improves the filling, but also that the second step of re-tanning process can be effectively replaced by exogenous collagen, contrary to the fatliquoring step (Test D). Moreover, when collagen is added at the end of the re-tanning process (Test C), a higher leather thickness increase is observed, tripled compared to the control, probably due to the presence of tannins that enhances the cross-linking with collagen (Chen et al. 2001).

Table 1 Thickness of leather samples and filling efficiency

	Thickness (mm)		Filling efficiency (ΔT%)
	Before	**After**	
Test A	0.54 ± 0.02	0.78 ± 0.04	44
Test B	0.55 ± 0.03	0.81 ± 0.02	47
Test C	0.54 ± 0.03	0.96 ± 0.04	78
Test D	0.54 ± 0.04	0.59 ± 0.03	9
Test E	0.54 ± 0.02	0.82 ± 0.02	52
Control	0.55 ± 0.02	0.69 ± 0.03	25

Microscopy analysis

To visualize the effects of re-tanning processes on leather at different scales, sections and surfaces of tests and control samples were analyzed through a stereomicroscope and by a scanning electron microscope (Fig. 2).

The microscopic characteristics of samples, summarized in table 2, suggest that the fatliquoring step is needed as the first step of the re-tanning process both to obtain a homogeneous matrix and to allow the filling agents to act (Tests B, C, E and control), resulting Test A and Test D non-homogeneously filled. Moreover, although the filling efficiency of Test B and Test E are comparable, samples appear very different: the addition of collagen before the synthetic tannins and resins (Test B) improves the filling efficiency and therefore the quality of the leather surface, but it has not significant effect on the matrix, since the section results tight, similarly to the control. On the other hand, using of collagen at the end of the re-tanning process, both as additive (Test C) and substitutive filler (Test E), results in well-filled and homogeneous leather with open pores and uniform and smooth surface.

Test C and Test E, therefore, were selected as the best conditions for the application of collagen as additive or substitute filler during the re-tanning process, respectively.

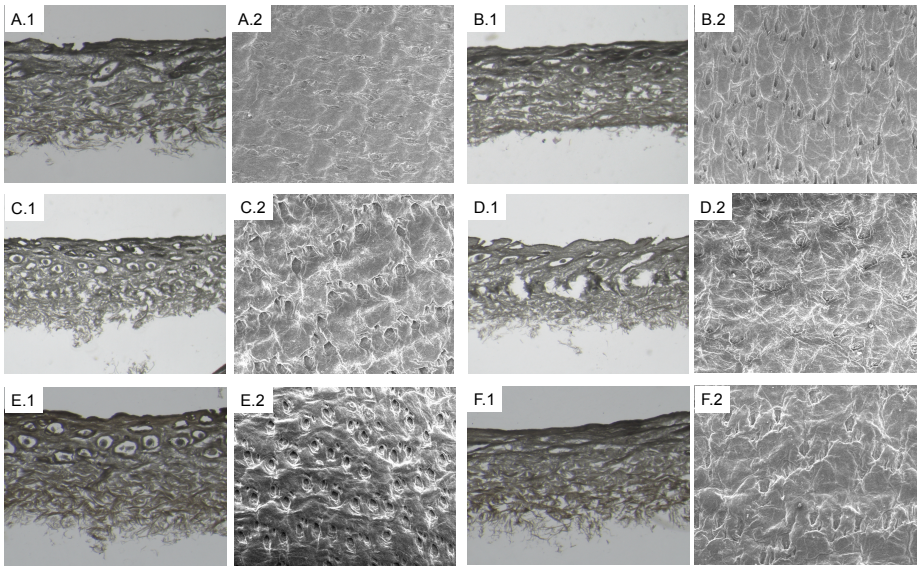


Fig. 2 Microscopy analyses of A) Test A, B) Test B, C) Test C, D) Test D, E) Test E and F) control sample

Table 2 Microscopic characteristics of sections and surfaces of filled leather

	Filling efficiency (ΔT%)	Microscopic characteristics	
		Section	Surface
Test A	44	• Inhomogeneous • Tight • Void areas	• Uniform • Closed pores • Wrinkled
Test B	47	• Homogeneous • Tight • No void areas	• Non-uniform • Open pores • Smooth
Test C	78	• Homogeneous • Full • No void areas	• Uniform • Open pores • Smooth
Test D	9	• Inhomogeneous • Loosened • Void areas	• Non-uniform • Closed pores • Wrinkled
Test E	52	• Homogeneous • Full • No void areas	• Uniform • Open pores • Smooth
Control	25	• Homogeneous • Tight • No void areas	• Non-uniform • Closed pores • Wrinkled

Cross-linking distribution

ATR-IR analyses were performed to evaluate the homogeneity of the filling: the spectra of different areas of Test C, Test E and control sample were collected and compared (*Online Resource 1: ATR-IR spectra of Test C, Test E and control sample*); cross-linking degrees were then measured as the ratio between the amide I and amide A signals (A_I/A_A) (Danylkovych et al. 2016). The amide A is characteristic of $–NH_2$ group, while amide I corresponds to –NH group; the increase of amide I signal and the decrease of amide A signal indicate the conversion of $–NH_2$ in –NH groups, i.e. the formation of new covalent bonds, thus the ratio of these signals is directly correlated to the cross-linking degree, as reported by Albu et al. and Garcia et al. (Sommer et al. 2021).

The control sample has a lower cross-linking degree with the highest variability (0.78 ± 0.10) respect to the tests (0.89 ± 0.02 of Test C, 0.98 ± 0.01 of Test E), which indicates a weaker and inhomogeneous fixing of the filling agents in leather matrix, in fact when collagen is introduced in the re-tanning process, especially in the Test E that used the extracted collagen as substitute filler, the cross-linking degrees increased and conversely the standard deviations were reduced up to ten times, thus indicating the homogeneous distribution of the collagen in the leather matrix (*Online Resource 2: cross-linking degrees of Test C, Test E and control sample*).

Properties of filled leather

To evaluate the effect of collagen on the filled leather, mechanical properties (which describe the behavior of leather under the application of a load (Garcia et al. 2009; Albu et al. 2014), and organoleptic properties, such as silkiness, softness, smoothness, and any other pleasant touch feelings, were analyzed.

The mechanical properties were measured through physical analyses: as shown in Table 3, Test C and Test E exhibited better tensile and tear strength compared to control, moreover the elongation at the break also increased. Therefore, the employment of collagen directly enhances the physical properties of leather.

Table 3 Mechanical properties of control and test samples

	Control	Test C	Test E
Shrinkage temperature (°C)	> 95	> 95	> 95
Tensile strength (N/mm^2)	17.0	22.4	23.7
Elongation at break (%)	75	96	80

Tear strength (N)	25.0	32.0	26.0
Grain distension (mm)	10.3	11.0	11.1

The organoleptic properties, whereas, were evaluated by a panel test: 30 estimators assigned, in a blind test, five possible degrees of sensorial experience in terms of the pleasure at the touch and the fullness. From these parameters, Touch Pleasure Index and Fullness Index were calculated as the sum of the obtained values provided by the possible maximum value (Jean Serge et al. 2019), *(Online Resource 3: Touch Pleasure Index and Fullness Index, formula and degrees)*.

From blind test it follows that control sample and Test C exhibit comparable feel properties, while Test E results 30% more full and 20% more pleasant at the touch than control sample, probably due to the better penetration and compatibility of collagen to leather (Fig. 3).

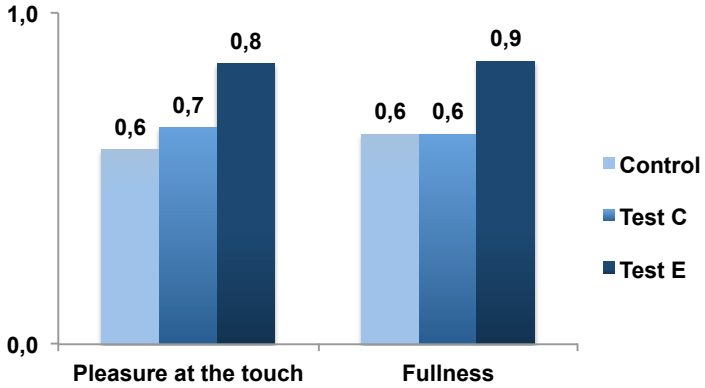


Fig. 3 Organoleptic properties of control and test samples

Finally, in order to study a correlation between sensorial and technical parameters, physical properties, cross-linking degree and organoleptic properties were reported in a Kiviat-like diagram, where the values obtained by mechanical tests were normalized by the maximum possible value for each parameter (Florio et al. 2015). The Figure 4 shows that higher cross-linking degrees correspond to higher tensile strength and better sensorial properties, while the elongation and the tear strength resulted not well correlated with the other parameters.

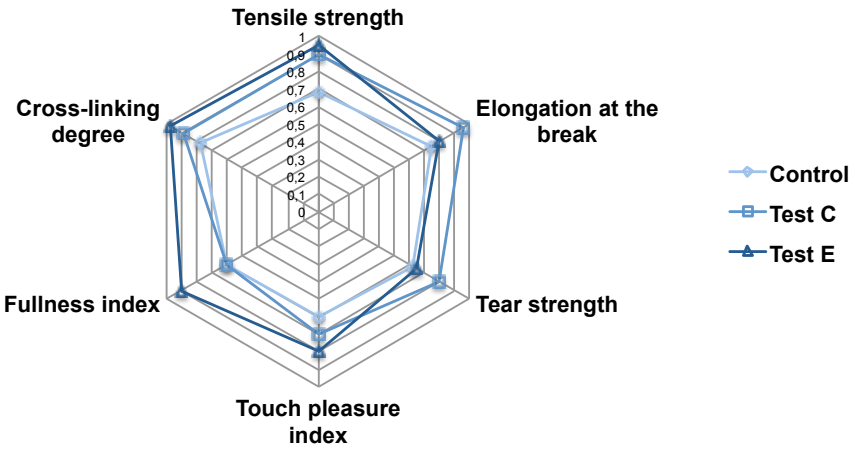


Fig. 4 Radar diagrams of organoleptic and, mechanical properties, and cross-linking degrees of control sample Test C and Test E

Costs/benefits analysis

According to its thickness, microscopic characteristics and physical and organoleptic properties, experts from local tanneries have currently evaluated the market value of ovine leather as *i)* 15.00 €/m^2 before the re-tanning (leather is characterized by veiny and loosen areas); *ii)* 25.00 €/m^2 after the traditional treatment of re-tanning (control), (leather is non-uniformly filled); and *iii)* 35.00 €/m^2 after the innovative collagen-based treatments (Test C and E), (leather is well-filled and uniform).

On the basis of this assessment, a costs/benefits analysis was evaluated for the leather product derived by our treatments *(Online Resource 4: costs/benefits analysis)*. The collagen-based procedure of Test C increase of 27% the process gain, even if the total costs of process result 35% higher than the ones related to the traditional re-tanning (Fig. 5). Regarding Test E, its processing rises the process gain up to 35%, despite being 25% more expensive than traditional re-tanning, rising. As a fact, as mentioned above, the filling step is crucial for the final aesthetic aspect of leather that have to provide uniform and well-filled leather after the re-tanning process, increasing the usable and, then, marketable area of each piece, thus rising the market value of each piece.

Considering the economic feasibility, the good properties that it confers to leather and, especially, the replacement of synthetic tannins and resins with a bio-based reactant, the re-tanning process of Test E acquires a double value: there is not only an economic advantage, but also an environmental one.

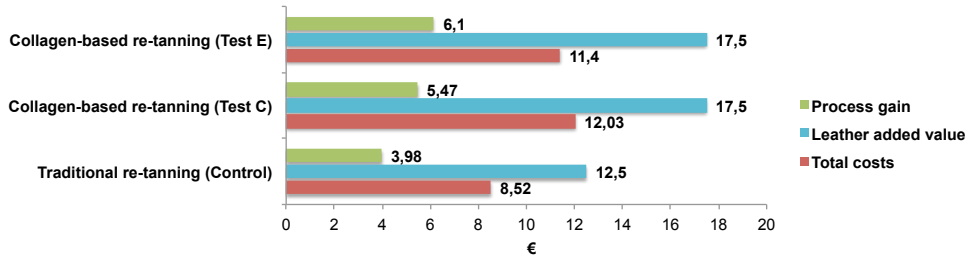


Fig. 5 Costs and benefits of traditional and innovative re-tanning procedures

Conclusions

In this study, extracted collagen from leather tanned solid wastes and casein were cross-linked *in situ* by a microbial transglutaminase during different steps of re-tanning process to fill the veiny areas of leather. Results show that extracted collagen can be applied as additive, as well as substitute filler: leather treated with collagen shows not only comparable mechanical properties but also better organoleptic properties than leather treated with synthetic fillers.

Using of an enzymatic cross-linker avoids the use of synthetic resins or fixatives, making leather processing more environmental sustainable and allows a more homogeneous distribution of the filling agent in the leather matrix.

The employment of collagen derived from leather solid wastes not only assures a very high compatibility between filling agent and leather matrix, but also adds value to leather scraps: the protein components of these wastes can be in this way applied to produce high quality leather, promoting cleaner production methods and circular production flow.

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Contributions

Conceptualization, investigation, methodology, validation, formal analysis, visualization, writing - original draft were performed by Marika Gargano. Validation, supervision, writing - review & editing were performed by Claudia Florio. Conceptualization, validation, visualization, supervision, project administration, writing - original draft, writing - review & editing were performed by Giovanni Sannia and Vincenzo Lettera.

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Clean Technologies and Environmental Policy
Focusing on Technology Research, Innovation, Demonstration, Insights and Policy Issues for Sustainable Technologies

Supplementary materials submitted

From leather wastes to leather: enhancement of low quality leather using collagen recovered from leather tanned wastes

A.1. Cross-linking degree

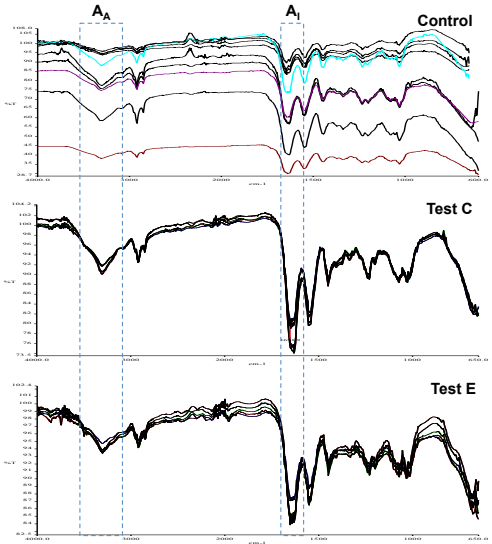

Figure A.1. ATR-IR spectra of different areas of control sample, Test C and Test E

Table A.1. Cross-linking degrees of control, Test C and Test E samples, expressed as the ratio between Amide I and Amide A signals (A_I/A_A)

	Control	Test C	Test E
A_I/A_A	0.81	0.92	0.98
	0.78	0.90	1.00
	0.75	0.92	0.97
	0.69	0.90	0.97
	0.65	0.89	0.99
	0.67	0.89	0.99
	0.96	0.88	0.98
	0.91	0.88	0.98
	0.83	0.87	0.98
	0.72	0.87	0.97
Average	0.78 ± 0.10	0.89 ± 0.02	0.98 ± 0.01

A.2. Touch Pleasure Index and Fullness Index

Touch Pleasure Index (TPI) and Fullness Index (FI) were calculated according to the following formula:

$$TPI\ or\ FI = \frac{d_1 + d_2 + d_3 + \cdots + d_n}{D \times n}$$

where d is each assigned degree; D is the maximum possible degree and n is the number of estimators that have performed the test.

Table A.2. Degrees of sensorial experience test

Degree	Pleasure at the touch	Fullness
1	Very unpleasant	Very empty
2	Unpleasant	Empty
3	Slightly pleasant	Slightly full
4	Pleasant	Full
5	Very pleasant	Very full

A.3. Costs/benefits analysis

Table A.3. Costs/Benefits analysis of traditional and innovative re-tanning processes

	Costs related to the re-tanning of 1 piece of ovine leather					
	Traditional re-tanning (CONTROL)		**Collagen-based re-tanning (TEST C)**		**Collagen-based re-tanning (TEST E)**	
	Input		**Input**		**Input**	
	Amount	Cost	Amount	Cost	Amount	Cost
Leather [a]	0.2 kg	€ 7.50	0.2 kg	€ 7.50	0.2 kg	€ 7.50
Process water [b]	0.0007 m^3	€ 0.0006	0.0007 m^3	€ 0.0006	0.0007 m^3	€ 0.0006
Fatliquoring step						
- Lipoderm [c]	0.006 kg	€ 0.024	0.006 kg	€ 0.024	0.006 kg	€ 0.024
- Coripol [d]	0.006 kg	€ 0.015	0.006 kg	€ 0.015	0.006 kg	€ 0.015
- Temperature [e]	45°C	€ 0.34	45°C	€ 0.34	45°C	€ 0.34
- Stirring [f]	100 rpm	€ 0.0135	100 rpm	€ 0.0135	100 rpm	€ 0.0135
- Process time	1 h	-	1 h	-	1 h	-
Process water [b]	0.0007 m^3	€ 0.0006	0.0007 m^3	€ 0.0006	0.0007 m^3	€ 0.0006
Resins-based filling step						
- Sellasol [g]	0.012 kg	€ 0.034	0.012 kg	€ 0.034		
- Melammina [h]	0.012 kg	€ 0.019	0.012 kg	€ 0.019		
- Tara [i]	0.012 kg	€ 0.028	0.012 kg	€ 0.028		
- Basytan [j]	0.012 kg	€ 0.022	0.012 kg	€ 0.022		
- Temperature [e]	45°C	€ 0.51	45°C	€ 0.51		
- Stirring [f]	100 rpm	€ 0.02	100 rpm	€ 0.02		
- Process time	1.5 h	-	1.5 h	-		
Collagen-based filling step						
- Collagen [k]			0.010 kg	€ 0.789	0.010 kg	€ 0.789
- Enzyme [l]			0.010 kg	€ 1.00	0.010 kg	€ 1.00
- Casein [m]			0.001 kg	€ 0.12	0.001 kg	€ 0.12
- Temperature [e]			45°C	€ 1.54	45°C	€ 1.54
- Stirring [f]			150 rpm	€ 0.06	150 rpm	€ 0.06
- Process time			4.5 h	-	4.5 h	-
Total costs	€ 8.52		€ 12.03		€ 11.40	
Leather added value [n]	€ 12.50		€ 17.50		€ 17.50	
Gain	€ 3.98		€ 5.47		€ 6.10	

a) Low quality leather costs 15.00 €·m^{-2}, 1 piece of ovine leather is 0.5 m^2 and 0.4 kg·m^{-2}
b) Water costs: 0.88 €·m^{-3} (Resolution ARERA n.665/2017/R/Idr)
c) Lipoderm costs: 4.030 €·kg^{-1}
d) Coripol costs: 2.551 €·kg^{-1}
e) Estimated as 1.27 kW per 0.27 €·kW^{-1}·h^{-1} per process time
Where 1.27 kW is the power required by the used oven (Heraus Function Line UT 6); 0.27 €·kW^{-1}·h^{-1} is the current cost of the electricity (Resolution ARERA n.621/2021/R/eel)
f) Estimated as 0.05 kW per 0.27 €·kW^{-1}·h^{-1} per process time
Where 0.05 kW is the power required by the orbital shaker (Stuart Scientific SSL1); 0.27 €·kW^{-1}·h^{-1} is the cost of electricity (Resolution ARERA n.621/2021/R/eel)
g) Sellasol costs: 2.863 €·kg^{-1}
h) Melammina costs: 1.660 €·kg^{-1}

i) Tara costs: 2.35 €·kg^{-1}
j) Basytan costs: 1.85 €·kg^{-1}
k) Collagen costs: 78.90 €·kg^{-1}. The extraction costs were estimated in "Leather industry towards circular economy: enzymatic extraction of potential high added-value products from tanned wastes"
l) Transglutaminase costs: 100.00 €·kg^{-1} (the cheapest price present on the market related to the product with analogue characteristics)
m) Casein costs: 120.00 €·kg^{-1} (the cheapest price present on the market related to the product with analogue characteristics)
n) In the same category, filled leather costs 25.00 €·m^{-2} and well filled leather 35.00 €·m^{-2}

coatings

MDPI

March 2023, submitted

From leather wastes to leather: development of new bio-based finishing system

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Abstract

Leather industry is, nowadays, hanging in the balance of two opposite roads: on one hand, the recent trend of legislation regarding the eco-sustainability of industrial processes is driving leather manufacturing processes towards the development of cleaner production methods; on the other hand, the widespread of new materials alternative to leather is needing the continuous increase of competitiveness of leather industry to develop new innovative and high-quality products.
Regarding the quality of products, one of the most crucial steps is the finishing which is responsible of the final aspect of leather. This step is also the one that most suffers of the use of pollutant chemicals, such as volatile organic compounds, potentially toxic cross-linkers and hardly-biodegradable resins. In this perspective, developing a finishing formulation able to confer to leather durability and quality properties requested by the market and, at the same time, that follows an eco-sustainable direction is one of the most challenging issues for the innovation of leather industry.
The aim of the present work is to propose a new finishing formulation not only in terms of a greener technology but also of a circular production flow, by recovering leather solid wastes. The developed finishing system is based on the application of collagen, extracted from tanned wastes through an enzymatic treatment, to cross-link and bind to the leather surface. The new bio-based finished leather was compared to a resins-based finished leather showing the same quality standards requested by the market.

Chapter 2

Introduction

Leather finishing is the last step of the leather manufacturing processes; it is responsible of the final properties and aesthetic characteristics of leather: hydrophobicity, color fastness, rub resistance, gloss and color homogeneity; properties which are directly linked to the composition of finishing formulations [1,2].

Finishing involves two coating steps: base coat and top coat. Base coat mainly consists in the application of solvents and binders able to interact with the surface of crust leather, thus modifying its chemical properties and allowing fixing of pigments and dyes. Top coat provides protection of the finished leather surface through the employment of lacquers [3]. To obtain high performances, base and top coats need to be high compatible: base coat, indeed, acts like a bridge between leather and top coat.

Finishing formulations comprises also film-forming agents, which can be divided into resins-based and protein-based formulations. The first group assures high performances in terms of color fastness and hydrophobicity, due to the plastic layer that coats leather; moreover, this process make use of the most widespread and effective finishing formulations, which usually contain polluting and toxic compounds [1].

The second group confers to leather a more natural aspect, a requisite required by high quality leather products. In this group, casein is the most used and well-known material. Casein finished leather is characterized by brightness and thermostability, but suffers of low water resistance and rub fastness [4].

Several methods to improve the properties of protein-based formulations have been developed, mainly introducing cross-linking agents that increase the film-forming ability of the used proteins, [5].

As a result, the current technologies for leather finishing need to be innovated both to satisfy the increasing market demand in terms of quality of the product and to respect the stringent legislation: reduction of the use of polluting and hazard chemicals [6–9].Leather industry is, therefore, aiming to develop new bio-based systems for finishing with the purpose to make leather process greener.

Developing new bio-based finishing systems, which have high compatibility between base and top coats and, as well as, between finishing formulations and leather surface, is the most challenging objective in this field [10].

In this perspective, collagen-based formulations are of interest being collagen highly compatible with leather [11] and, able to confer to leather resistance without affecting its natural aspect. Moreover, to be applied as finishing agent, collagen does not require a high purity

degree, thus it can be isolated from a wide range of sources. In the viewpoint of circular production flow, collagen derived from leather solid wastes can be effectively used in finishing formulations [12].
In our previous work [13], leather tanned solid wastes were enzymatically treated to extract collagen; the latter proved to be of high quality and highly compatible with leather, thus being able to fill loose parts of leather during the re-tanning process [14].
In the present study, collagen extracted from tanned leather wastes is used as an ingredient of base and top coats in leather finishing. The formulations were firstly prepared as films to analyze their properties and then spayed on leather. The developed formulations were compared to a top-in-the-range resins-based finishing formulation, in terms of physical properties, solutions and leather biodegradability.

Keywords: Circular Economy, enzymatic cross-linking, collagen-based coating, waste recovery, green industry, biodegradability

Materials and methods

Materials

Vegetable tanned bovine shavings, organic tanned bovine shavings and mineral tanned bovine shavings were collected from the Italian Leather Research Institute (form Veneto district, Italy).
Bovine crust leather furnished by A3 Leather Innovation Center (Igualada, Spain).
Casein from bovine milk was purchased from Sigma-Aldrich (Milan, Italy). Microbial transglutaminase Activa WM was furnished by Ajinomoto (Hamburg, Germany).
Luron Binder U, Melio Resin A-943, Aqualen Top 2006, Aqualen Top DC-2060, Metallic dye and Pigment were furnished by Stahl (Parets del Vallès, Spain).
Silicon was furnished by Pielcolor (Parets del Vallès, Spain).

Extraction of collagen

Vegetable tanned bovine shavings, organic tanned bovine shavings and mineral tanned bovine shavings were treated with enzymatic treatment to extract collagen as previously reported [13].

Films formation

Aqueous solutions of extracted collagen and casein (total solids value 30%) were prepared by varying the collagen/casein ratio (0:1, 1:1, 2:1, 5:1, 10:1 and 1:0) and by adding 0 or 50 U/g of transglutaminase. Consequently, 5 mL of the solutions were cast into a silicon plate (5×3 cm) and air-dried in a ventilated incubator at room temperature for 48 h. A Softness Tester (ST300 IUP/36, SDL Atlas, Sud Carolina, USA) was used to test the hardness of films.

BOD and COD analyses

The determination of the chemical oxygen demand (COD) and biochemical oxygen demand (BOD) was performed according the UNI EN ISO 6060:1986 and UNI EN ISO 5815-1, respectively.

Application of finishing formulations

The finishing formulations are shown in Table 2 and 3. Leather samples were sprayed twice with the base coat formulations using a spray gun, dried at 40°C in a heater, and then pressed at 70°C, 100 kg/cm^2, 2 seconds. The base coat was applied and dried again twice. Leather samples were, then, sprayed twice with the top coat formulations, dried at 40°C in a heater, and then pressed at 75°C, 100 kg/cm^2, 2 seconds. Finished leather was stored for 5 days to let the finishing settle.

Mechanical tests

Mechanical tests were performed as follows:

- Softness according to UNI EN ISO 17235:2015
- Color fastness to water spotting according to UNI EN ISO 15700:1998
- Color fastness to artificial light according to UNI EN ISO 105-B02
- Color fastness to rubbing UNI EN ISO 11640:2018

Biodegradability analysis

Leather biodegradability was analyzed according to UNI EN ISO 20136:2020.

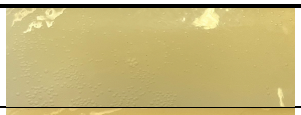
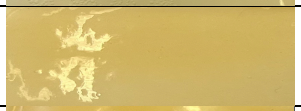
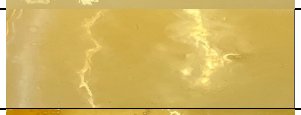
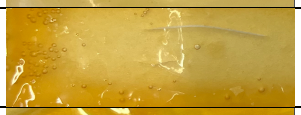
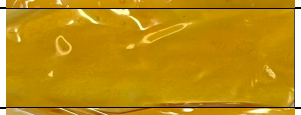
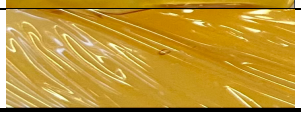
Results and discussion

Films formation

In our previous studies, the enzymatic mediated cross-linking between collagen and casein was optimized [13] and the applicability of collagen as a filler in leather processing was assessed [14]. Since the promising results of the application of collagen in leather processing, collagen extracted from leather solid wastes was used as ingredient for finishing formulations. Therefore, collagen-based films were developed to assess their applicability for leather finishing. Transglutaminases (TGase) were used as cross-linking bio-agent, instead of chemical ones, to obtain homogeneous films. Hardness of the films was measured according the Shore scale A, whose values are commonly used to report the hardness of materials in a range from 0 to 100, where 0 and 100 are related to extra-soft and extra-hard materials, respectively.

Different collagen/casein concentration ratios were investigated to study their influence on films properties and the condition 3 of Table 1 was selected, on the basis of the lower Shore value.

Table 1. Properties and hardness of films obtained incubating collagen and casein (0:1, 1:1, 2:1, 5:1, 10:1, 1:0) with 50 U/g transglutaminase

	Film	Collagen:Casein	Shore scale A	Properties
1		0:1	85	• Transparent • Glossy • Hard
2		1:1	75	• Transparent • Glossy • Hard
3		2:1	65	• Transparent • Glossy • Medium-hard
4		5:1	85	• Transparent • Glossy • Hard
5		10:1	>90	• Transparent • Glossy • Extra-hard
6		1:0	>90	• Transparent • Glossy • Extra-hard

Collagen-casein (2:1) solution was selected to replace the synthetic resins in a top-of-the-range finishing formulation (Table 2-3); thus, films of the finishing agents were developed (in proportion to each other as reported in the tables 2 and 3).

Table 2. Base coat formulations

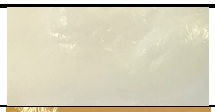
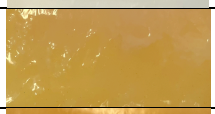
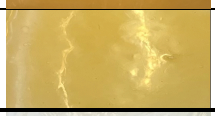
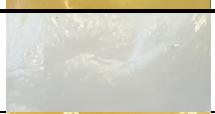
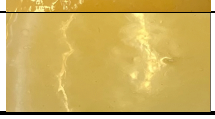
Base coat							
BC (Control)		**BC1**		**BC2**		**BC3**	
Component	**%**	**Component**	**%**	**Component**	**%**	**Component**	**%**
Water	28	Water	28	Water	28	Water	28
IPA	2	IPA	2	IPA	2	IPA	2
Polyurethane 1	34	**Collagen-casein**	34	Polyurethane 1	34	**Collagen-casein**	52
Acrylates	18	Acrylates	18	**Collagen-casein**	18		
Silicon	2	Silicon	2	Silicon	2	Silicon	2
Pigment	16	Pigment	16	Pigment	16	Pigment	16

Table 3. Top coat formulations

Top coat							
TC (Control)		**TC1**		**TC2**		**TC3**	
Component	**%**	**Component**	**%**	**Component**	**%**	**Component**	**%**
Water	38	Water	38	Water	38	Water	38
Polyurethane 1	30	**Collagen-casein**	30	Polyurethane 1	30	**Collagen-Casein**	47
Polyurethane 2	17	Polyurethane 2	17	**Collagen-casein**	17		
Silicon	15	Silicon	15	Silicon	15	Silicon	15

From the data concerning the films properties (table 4), it clearly results that solutions containing both collagen and casein are compatible with both polyurethanes and acrylates, since all films appear homogeneously distributed; when collagen and casein are added to polyurethanes, finishing films get a more appreciable transparency, a valuable property in leather finishing; moreover, films based only on collagen and casein show appealing properties even if the hardness increases.

Table 4. Properties and hardness of films obtained incubating resins and collagen and casein (2:1) with transglutaminase according to table 2 and 3

	Film	Composition	Shore Scale A	Properties
BC		Polyurethane 1 – Acrylates	50	• Non-transparent • Matt • Medium-soft
BC1		Polyurethane 1 – Collagen/casein	55	• Transparent • Glossy • Medium-soft
BC2		Collagen/casein – Acrylates	60	• Non-transparent • Matt • Medium-hard
BC3		**Collagen/casein**	65	• Transparent • Glossy • Medium-hard
TC		Polyurethane 1 – Polyurethane 2	60	• Non-transparent • Glossy • Medium-hard
TC1		Collagen/casein – Polyurethane 2	70	• Transparent • Glossy • Medium-hard
TC2		Polyurethane 1 – Collagen/casein	55	• Transparent • Glossy • Medium-soft
TC3		**Collagen/casein**	65	• Transparent • Glossy • Medium-hard

Biodegradability

As reported in our previous work [13], collagen extracted from leather solid wastes presents several contaminants, such as metals and polyphenols, thus, to assess its environmental impact, the biodegradability of collagen and casein solutions was measured as ratio between the Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD), [15]. Comparing collagen-casein biodegradability to that of polyurethanes and acrylates used in the standard finishing formulation, it results that collagen-casein solutions are more biodegradable than resins solutions (Table 5), especially if compared to Polyurethane 2 and Acrylates that are classified in the toxic zone, according to the levels of biodegradability in Figure 1 [16].

Table 5. Biodegradability (BOD/COD ratio) of collagen-casein solution, Polyurethane 1, Polyurethane 2 and Acrylates of finishing formulations

Solutions	BOD/COD ratio	Biodegradability level
Collagen-casein	0.598	Easily biodegradable zone
Polyurethane 1	0.133	Non-biodegradable zone
Polyurethane 2	0.023	Toxic zone
Acrylates	0.025	Toxic zone

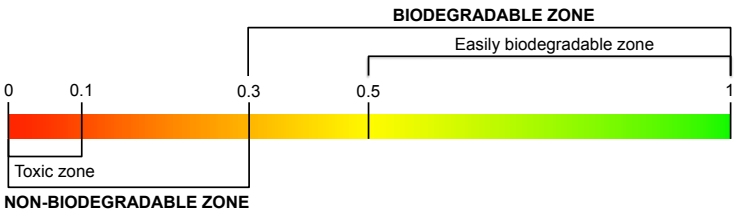


Figure 1. Levels of biodegradability

Application of collagen and casein in finishing formulations

Leather samples were treated combining each base coat with each top coat formulation (Table 2-3). The physical properties of the finished leather were measured and reported in Table 6.

Table 6. Physical properties of finished leather with standard and developed finishing formulations

Sample		Softness	Color fastness to			
			Rubbing* (Dry)	Rubbing* (Wet)	Artificial light	Water spotting*
1	**BC+TC**	2.7	5	5	>8	5
2	**BC+TC1**	2.0	5	1	>8	1
3	**BC+TC2**	2.0	5	1	<4	1
4	**BC1+TC**	2.0	5	4-5	>8	4
5	**BC1+TC1**	2.0	5	1-2	>8	2
6	**BC1+TC2**	1.8	5	1	<4	1
7	**BC2+TC**	1.8	5	5	>8	4
8	**BC2+TC1**	1.5	5	2	>8	3
9	**BC2+TC2**	1.3	5	5	<4	5
10	**BC3+TC3**	1.6	5	5	>8	5

*Mark 1 indicates very poor fastness and mark 5 indicates very good fastness

All samples exhibit a very valuable color fastness to rubbing in dry conditions, but only samples 1, 4, 7 and 10 maintain the same values in wet conditions. Furthermore, as far as the sample 10 is concerned, whose resins are completely replaced by collagen and casein all its physical properties, but the softness, are comparable to sample 1 (standard formulation), probably because of the high compatibility between base and top coats.

The macroscopic aspect of sample 10 (Fig. 2b), however, did not meet certain quality standards: it shows white spots on the surface, possibly caused by the accumulation of salts present in the extracted collagen stock solution [13].

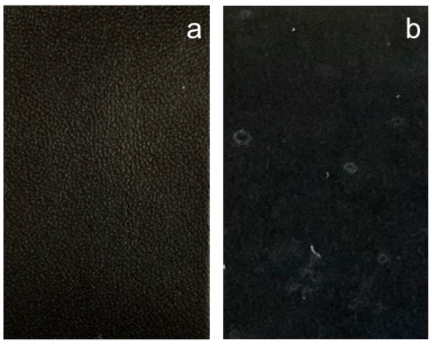


Figure 2. Finished leather surface of a) sample 1 and b) sample 10

Since its very suitable physical properties achieved without adding any resins to the finishing treatment, sample 10 (BC3+TC3) was object of further investigation. Formerly, in order to avoid the formation of white spots on leather surface, the total solids content (TSC) of collagen-casein solution was gradually decreased. Panels d, e, f of Figure 3, point out that through decreasing the total solids content in the treatment under than 20%, no formation of white spots occurred.

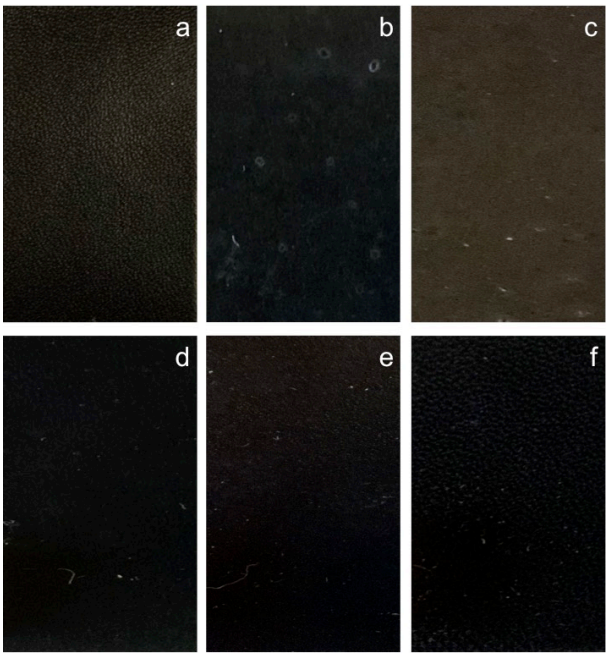


Figure 3. Finished leather surface with a) BC+TC; b) BC3 (30%TSC) +TC3 (30%TSC); c) BC3 (20%TSC) +TC3 (20%TSC); d) BC3 (12%TSC) +TC3 (7%TSC); e) BC3 (10%TSC) +TC3 (7%TSC) and f) BC3 (7%TSC) +TC3 (10%TSC)

Looking at the physical properties of all samples (Table 7), the total solid content resulted affecting color fastness too. In particular, when it is dosed at a concentration lower than 12% in the base coat (samples E and F), color fastness to wet rubbing and water spotting decreases. Therefore, sample D treated by 12% and 7% of base and top coats, respectively, was selected because of the absence of white spots on the surface and of its physical properties, comparable to those of the reference (sample A - standard formulation).

Table 7. Physical properties of leather treated with a) BC+TC (standard formulation), b) BC3 (30% TSC) + TC3 (30% TSC), c) BC3 (20% TSC) + TC3 (20% TSC), d) BC3 (12% TSC) + TC3 (7% TSC), e) BC3 (10% TSC) + TC3 (7% TSC) and f) BC3 (7% TSC) + TC3 (12% TSC)

Sample	Softness	Color fastness to			
		Rubbing* (Dry)	Rubbing* (Wet)	Artificial light	Water spotting*
A	2.7	5	5	>8	5
B	1.6	5	5	>8	5
C	1.7	5	5	>8	5
D	1.9	5	5	>8	5
E	1.8	5	3-4	>8	3
F	1.9	5	1-2	>8	1

*Mark 1 indicates very poor fastness and mark 5 indicates very good fastness

To increase the softness of the leather treated with this improved bio-based formulation, the concentration of enzyme, related to the cross-linking solution, was decreased from 50 U/g of the initial formulation to 5 U/g, thus reducing the cross-linking degree between collagen and casein.
Decreasing amount of enzyme used, indeed, increases softness, but when the amount of transglutaminase is lower than 15 U/g, the color fastness severely worsens (Table 8).

Table 8. Physical properties of finished leather with a) BC+TC; b) BC3 (12% TSC) + TC3 (7% TSC) 50 U/g TGase; ; c) BC3 (12% TSC) + TC3 (7% TSC) 25 U/g TGase; ; d) BC3 (12% TSC) + TC3 (7% TSC) 15 U/g TGase; ; e) BC3 (12% TSC) + TC3 (7% TSC) 10 U/g TGase; ; f) BC3 (12% TSC) + TC3 (7% TSC) 5 U/g TGase

		Color fastness to			
	Softness	**Rubbing* (Dry)**	**Rubbing* (Wet)**	**Artificial light**	**Water spotting***
A	2.7	5	5	>8	5
B	1.9	5	5	>8	5
C	2.3	5	5	>8	5
D	2.5	5	5	>8	5
E	2.5	5	4	6-8	3-4
F	2.8	4-5	1-2	4-6	2-3

*Mark 1 indicates very poor fastness and mark 5 indicates very good fastness

The selected finishing formulation (BC3 (12% TSC) + TC3 (7% TSC) + 15 U/g of TGase) allows to fabricate leathers with the same surface properties of the standard formulation (Fig. 4), according to the quality criteria reported in the UNI EN ISO 10826:2020.

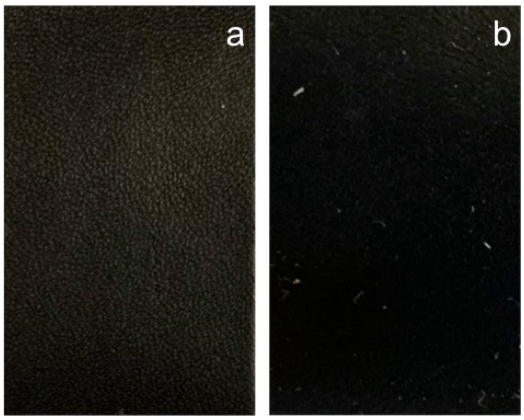


Figure 4. Finished leather surface with a) standard finishing formulation (BC+TC) and b) developed finishing formulation (BC3 (12% TSC)+TC3 (7% TSC) + 15U/g TGase)

Conclusions

The reduction of chemicals and the valorization of solid wastes are challenging and complex issues for the transition of leather industry towards cleaner production. In the present study, collagen extracted from leather solid wastes were used for developing a new bio-based finishing formulation.

The developed system allows the replacement, in finishing operations, of recalcitrant polyurethanes and acrylic resins with waste-extracted collagen by its enzymatic cross-linking with casein.

Physical tests of softness and color fastness proved that the present formulation results innovative and eco-sustainable and confers the same quality standards to leather of a top-of-the-range resins-based formulation.

CRediT authorship contribution statement

Marika Gargano: Conceptualization, Investigation, Methodology, Validation, Formal analysis, Visualization, Writing - original draft. **Anna Bacardit**: Validation, Supervision, Writing - review & editing. **Giovanni Sannia, Vincenzo Lettera**: Conceptualization, Validation, Visualization, Supervision, Project administration, Writing - original draft, Writing - review & editing.

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CONCLUSIONS

Conclusions

The present PhD project aimed to develop biotechnological approaches for the transition of leather industry towards cleaner production processes and circularity. These objectives have been successfully reached through the recovery and valorization of leather solid wastes by the extraction of collagen and its application in leather processing.

The first objective, aimed to the recovery of leather solid wastes, has been matured through the development of new cost-effective enzymatic methods for collagen extraction, allowing to obtain high quality collagen, to reduce the amount of used chemicals and to increase the extraction yields.

Regarding the second objective, through its enzymatic cross-linking with casein, the extracted collagen has been employed back in leather processing as filling and finishing agent. The re-design of re-tanning and finishing protocols allowed to develop green systems for the most polluting leather processing steps and to obtain leather products with characteristics comparable, if not better, to the ones produced by conventional processes.

APPENDIX

Poster

Marika Gargano, Claudia Florio, Marco Nogarole, Vincenzo Lettera, Giovanni Sannia. "**Circular Economy in the leather industry: recovery and valorization of wastes from wet-white shavings**". *IFIB2020 - International Forum on Industrial Biotechnology and Bioeconomy*, Rome, 1st - 2nd October 2020.

Conference proceeding

Claudia Florio, Marika Gargano, Vincenzo Lettera, Ilaria Pagliuca, Marco Abbro, Giovanni Sannia. "**Tanning biotechnologies for novel sustainable and circular materials**". *III IULTCS EuroCongress*, Vicenza, 18th – 20th September 2022.

Publications

P1- Marika Gargano, Claudia Florio, Vincenzo Lettera, Giovanni Sannia. "**Biotechnological approaches for valorization of leather solid wastes: recovery and applications of collagen**", 2021. *Cuoio Pelli Materie Concianti*, 97, 49–52.

P2- Marika Gargano, Vincenzo Lettera, Giovanni Sannia. "**Chimica Verde, Economia Circolare e Biotecnologie: una nuova linfa per il futuro dell'industria italiana della pelle**", 2022. *La Chimica e l'Industria*, submitted.

P3- Marika Gargano, Claudia Florio, Angela Amoresano, Giovanni Sannia, Vincenzo Lettera. "**Leather industry towards circular economy: enzymatic extraction of potential high added-value products from tanned wastes**", 2023. *Journal of Environmental Management, in press*.

P4- Marika Gargano, Claudia Florio, Giovanni Sannia, Vincenzo Lettera. "**From leather wastes to leather: enhancement of low quality leather using collagen recovered from leather tanned wastes**", 2023. *Clean Technologies and Environmental Policy*, submitted.

P5- Marika Gargano, Anna Bacardit, Giovanni Sannia, Vincenzo Lettera. "**From leather wastes to leather: development of new bio-based finishing system**", 2023. *Coatings*, submitted.

Experience in foreign laboratory

1st September 2021 – 30th April 2022:
Visiting PhD student in the laboratory of prof. Anna Bacardit at A^3 Leather Innovation Center of the Univeristy of Lleida in Igualada.
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TECNOLOGIE ABILITANTI PER NUOVI MATERIALI CIRCOLARI

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Approcci biotecnologici per la valorizzazione degli scarti solidi dell'industria: il recupero e l'applicazione del collagene

La lavorazione del cuoio è un tema di estremo interesse nel campo dell'Economia Circolare; consiste principalmente, infatti, in attività di recupero e valorizzazione di uno scarto dell'industria alimentare (la pelle animale che proviene dalla macellazione).
La conversione di pelli grezze in cuoio implica diverse fasi di processo che producono significative quantità di rifiuti. Basti pensare che la produzione 200 kg di cuoio necessiti una tonnellata di pelle grezza, i cui restanti 800 kg sono smaltiti come scarti solidi composti principalmente da pelli e semilavorati, senza contare l'enorme quantità di acqua utilizzata e scaricata come refluo.
Considerata la potenziale presenza di inquinanti, lo smaltimento di questi scarti può determinare un impatto ambientale negativo, nonché ricadute sul piano economico.
Negli ultimi anni, infatti, è stato promosso lo sviluppo di processi che mirano a ridurre l'uso di composti inquinanti e tossici, che non sono tuttavia sufficienti per risolvere le criticità riguardanti l'ambiente, considerando ad esempio, la concia vegetale che se da un lato riduce l'impiego di sostanze potenzialmente impattanti, dall'altro scarica grandi quantità

Biotechnological approaches for valorization of leather solid wastes: recovery and applications of collagen

Leather processing is an issue of extreme interest in the field of Circular Economy; in fact, it mainly consists in activities of recovery and enhancement of a waste of the food industry (raw animal skin that comes from slaughter).
Converting raw hides in leather involves several process steps that produce a huge amount of wastes. Suffice it to say that the production of 200 kg of leather requires a ton of raw hides and the remaining 800 kg are disposed as solid wastes, mainly composed by hides and semi-processed skins, not to mention the massive amount of water used and discharged as wastewater.
Given the potential presence of pollutants, the disposal of these wastes can be responsible for a negative environmental impact, as well as for possible economic issues.
In recent years, the development of processes aimed at reducing the use of polluting and toxic compounds has been promoted, but these are not sufficient to solve all environmental issues, considering, as an example vegetable tanning, which on the one hand reduces the use of potentially impacting substances, on the other hand discharges large quantities of vegetable tannins which degra-

di tannini vegetali che, degradandosi molto lentamente, persistono per lungo tempo nell'ambiente.
Inoltre, negli ultimi anni sono stati sviluppati molteplici approcci di concia alternativa al cromo, con conseguente maggiore complessità nella definizione delle strategie per il trattamento e la valorizzazione degli scarti derivanti dalle pelli di nuova generazione esenti da cromo.
In questa prospettiva, lo sviluppo di nuove strategie volte a preservare l'ambiente deve essere accompagnato, in un quadro di economia circolare, dalla riduzione, recupero e riutilizzo degli scarti industriali, con lo scopo di chiudere il flusso circolare che caratterizza l'industria della pelle fin da principio.
Gli scarti solidi di cuoio sono composti per circa il 30% da proteine, rappresentate per il 90-95% da collagene. I metodi convenzionali di recupero del collagene dagli scarti di pellame si basano sull'estrazione chimica: agenti acidi o alcalini possono agire sulla matrice del pellame e rilasciare il collagene.
Tramite queste procedure, il collagene ottenuto è completamente destrutturato e idrolizzato in piccoli polipeptidi (10-20 kDa), limitando la sua applicabilità principalmente in campo agricolo, come elemento per la formulazione di fertilizzanti.
Tuttavia, questo biopolimero si è dimostrato essere una molecola molto versatile e dipendentemente dalla fonte, dal metodo di recupero e quindi dalla qualità, il collagene può essere applicato in una vasta gamma di campi, dalla formulazione di farmaci e cosmetici alla medicina ricostruttiva.
In questo contesto si inserisce il presente progetto che mira alla valorizzazione degli scarti solidi derivanti dai processi di concia (che rappresentano più del 20% degli scarti solidi totali dell'industria conciaria) attraverso la loro conversione in prodotti ad alto valore aggiunto e, in particolare, sviluppando nuovi metodi per l'estrazione e il riutilizzo del collagene (Fig.1).
Sono, quindi, stati sviluppati approcci enzima-

de very slowly persisting for a long time in the environment. Furthermore, in recent years multiple chrome-free tanning approaches have been developed, however this causes greater complexity in defining strategies for the treatment and valorization of wastes deriving from this new generation chromium-free leathers.
In this perspective, the development of new strategies to preserve environment must be accompanied, within a Circular Economy framework, by the reduction, recovery and reuse of industrial wastes, with the purpose of closing the circular flow that characterizes the leather industry from the beginning.
Leather solid wastes are composed of about 30% protein, represented by 90-95% collagen. Conventional methods of collagen recovery from leather wastes are based on chemical extraction: acid or alkaline agents can act on the leather matrix and release collagen. Through these procedures, obtained collagen is completely deconstructed and hydrolyzed in small polypeptides (10-20 kDa), limiting its applicability mainly to the agricultural field, as additive in the formulation of fertilizers.
However, this biopolymer has proven to be a very versatile molecule and, depending on the source, recovery method and therefore quality, collagen can be applied in a wide range of fields, from cosmetics and drug formulation to reconstructive medicine.
In this context, the present project aims at enhancing solid wastes from tanning processes (which represent more than 20% of total solid wastes from the leather industry) through their conversion into high added value products and, in particular, by developing new economical and environmental viable methods for the extraction and reuse of collagen (Fig.1).
Enzymatic approaches for the extraction of collagen have been developed allowing, when compared to the traditional extraction methods, to: i) increase the amount of extracted collagen up to 10% (w/w); ii) reduce

tici per l'estrazione del collagene che hanno permesso, confrontati con i metodi di estrazione tradizionali, di: i) aumentare la quantità di collagene estratto fino al 10% (p/p); ii) ridurre il tempo di reazione (di almeno due volte); iii) ridurre l'uso di agenti chimici fino a 10 volte; e iv) ottenere un prodotto di alta qualità, ampliando quindi il suo spettro di applicazioni. I metodi di estrazione enzimatica risultano essere efficaci su scarti derivanti da diversi tipi di concia, indipendentemente dalla natura degli agenti concianti utilizzati.
Il collagene estratto è stato caratterizzato in termini di identificazione proteica, contenuto di polifenoli e metalli e pesi molecolari.
Le analisi di spettrometria di massa hanno provato che gli estratti sono prevalentemente composti da collagene, con tracce di metalli o polifenoli a seconda del metodo di concia che la materia prima ha subito. Inoltre, le analisi di spettroscopia infrarossa (FT-IR) hanno permesso non solo di confermare le informazioni ottenute, ma anche di valutare il grado di integrità del collagene (Fig.2). Gli spettri del collagene estratto via enzimatica sono paragonabili a quelli del collagene nativo, suggerendo che il processo di estrazione non infici la struttura del collagene, contrariamente a quanto accade durante il tradizionale processo di estrazione per via chimica.
Il collagene estratto è stato, quindi, sottoposto a ulteriori caratterizzazioni, quali l'analisi dei pesi molecolari e di microscopia a scansione elettronica (SEM), confermando che il collagene estratto conserva la sua struttura e che la sua qualità è tale da non rendere necessarie successive fasi di purificazione. Il collagene estratto mostra, infatti, le caratteristiche tipiche del collagene nativo, come le proprietà gelificanti.
L'alta qualità del collagene estratto ha permesso di sviluppare strategie di cross-linking e funzionalizzazione con lo scopo di ottenere un prodotto applicabile sia nell'industria conciaria sia nella stampa 3D.
La capacità del collagene di formare cross-lin-

the reaction time (at least two times); iii) reduce use of chemical agents up to 10 times; and iv) recover a high quality product, thus expanding its range of applicability. The developed enzymatic extraction methods resulted effective on wastes from different types of tanning, regardless of the nature of the tanning agents used.
The recovered collagen has been characterized in terms of protein identification, polyphenols and metals content and molecular weights.
Mass spectrometry analyses have shown that the extracts are mainly composed of collagen, with traces of metals or polyphenols depending on the tanning method that the raw material has undergone. In addition, infrared spectroscopy analysis (FT-IR) not only confirmed the information obtained, but also assessed the degree of integrity of collagen (Fig.2). The spectra of collagen extracted by the enzymatic process are comparable to those of the native collagen, suggesting that the extraction process does not affect the structure of collagen, contrary to what happens during the traditional chemical extraction process.
The extracted collagen was further characterized by molecular weight and scanning electron microscopy (SEM) analyses, confirming that it preserves an intact structure (Fig.3) and that its quality is such that no subsequent purification step is needed. As a fact the extracted collagen displays the typical features of native collagen, such as gelling properties.
The high quality of the extracted collagen allowed the development of cross-linking and functionalization strategies in order to obtain products applicable both in the tanning industry and in 3D printing. The ability of collagen to cross-link with casein (in an enzymatically catalyzed reaction) allows, in fact, its application as a filling or finishing agent for low-quality leather.
In 3D printing collagen is considered a preferential material for the production of bio-inks.

ks con la caseina (in una reazione catalizzata da enzimi) permette, infatti, la sua applicazione come agente di filling e di rifinizione delle pelli di bassa qualità.
Nel campo della stampa 3D il collagene è considerato un materiale di predilezione per la produzione di bio-inchiostri. Lo sviluppo di protocolli di derivatizzazione del collagene estratto con l'anidride metacrilica, permettendo la formazione di cross-links mediati dai raggi UV, produce un materiale di partenza ideale per la generazione di inchiostri per la stampa 3D.
In conclusione, il trattamento enzimatico degli scarti di pellame risulta essere una strategia alternativa valida per poter recuperare collagene di alta qualità e conseguentemente per permetterne l'applicazione in diversi ambiti industriali.

The development of derivatization protocols of the recovered collagen with methacrylic anhydride, allowing the formation of UV-mediated cross-links, produced an ideal starting material for the production of 3D inks.
In conclusion, the enzymatic treatment of leather solid wastes turns out to be a valid alternative strategy to recover collagen of high quality and consequently to allow its application in different industrial fields.

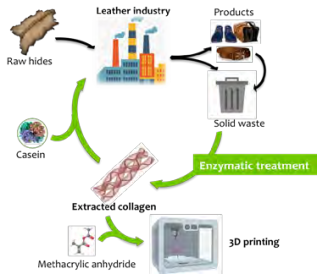

Fig.1: Graphical abstract of the project

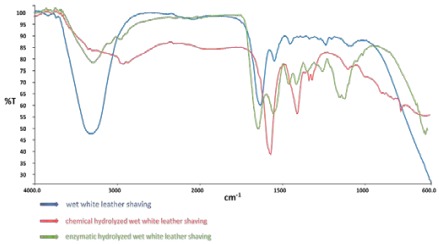

Fig.2: ATR-IR spectra overlap of differently treated and not treated samples

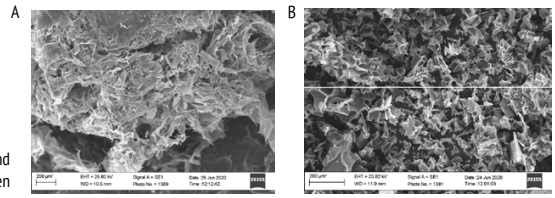

Fig.3: SEM images of native (a) and extracted (b) collagen

Tanning biotechnologies for novel sustainable and circular materials

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Abstract

The Circular Economy principles are well integrated with leather production processes and with the current direction that tanning chain is following, that is becoming even more strategic as a model of development for sustainable growth; today the tanning sector has an impacting role as part of the Bioeconomy scenario, indeed.

On the other hand, the production of solid tanning waste is a critical issue to be addressed as a new strategic challenge, aimed at fostering the transformation of the international economy in a greener, more resilient and circular system. These wastes can represent a great opportunity for the development of new products and applications in different fields, however their valorisation can be affected by some critical issues, mainly connected to the presence of chemical components deriving from the production process (metal iones and/or organic tanning agents, biocides, synthetic polymers, etc.), that currently limits the possible use of wastes in some applications.

This work aims, on the one hand, to minimize the use of traditional chemicals in the production process and, on the other hand, to use sustainable biotechnological approaches for the use of wastes in the production of new generations of bio-based circular materials.

Two main biotechnological approaches were particularly considered; one which involves the selection and use of enzymatic systems for obtaining collagen hydrolysates with high added value, for its use both in the tanning process, as filler and as finishing agent, than in the production of novel bioinks in 3D printing technologies. In this approach, ad hoc enzymes were selected for the treatment of trimming and shaving of wet-white wastes, derived from different tanning systems; another approach involved the use of symbiotic cultures of bacteria and yeasts for the production of a cellulose film and its use in the creation of bio-based finishing layers.

Both approaches provide important tools to produce new generation of circular and sustainable materials.

Keywords**: *circular materials; circular finishing; sustainable finishing; biotechnological approaches; leather waste valorisation; bio-based fillers; bio-based polymers.***

1. Introduction

Leather is one of the leading natural-based materials among the fashion and luxury supply chain; its natural and ancient origin, its high performances and qualities, guarantee a unique international leadership to the tanning industry. Leather is also a circular material, deriving from the transformation of a food industry by-product, raw leather; the purpose of the tanning process, consisting in a series of chemical and mechanical treatments, is to make the skin non-perishable, stable, resistant over time, in order to guarantee a durable and high value-added finished product, where, in recent years, many efforts have been made to improve the sustainability content of leathers, mainly through the experimentation of novel green solutions for tanning.

In addition, the leather supply chain is a crucial sector of the bioeconomy, being the most driving part of the textile and fashion industry in this area. The bioeconomy is a particularly strategic and currently remarkable sector and can be defined as the socio-economic system associated with the enhancement of renewable biological resources, for their use in the production of other goods, materials and products. Bioeconomy is closely connected to the concept of circular economy, especially with reference to the extensive use of agricultural waste and by-products of the food industry as an input for the creation of products with greater added value; bioeconomy is also intrinsically circular where carbon is sequestered from the atmosphere by plants, reused in natural transformations and recycled as soil carbon (Gowdy J. M.,et al., 1998; Leipold S.,et al., 2018).

In order to improve the sustainability and circularity content of leather products, it is necessary to foster the exploration of progressively more advanced and innovative scientific and technological approaches. Particularly interesting and promising in this sense are the approaches based on enabling technologies and particularly on Biotechnologies.

According to the definition deriving from the Convention on Biological Diversity (UNITED NATIONS 1992), "Biotechnology" means any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use. The term in the plural form (Biotechnologies) is currently more commonly used to indicate the plurality of technologies developed and the related fields of application.

Specifically, the activities of this work concern the use of innovative and sustainable approaches for the study, characterization and transformation of biological systems of tanning interest, including the engineering and innovative use of enzymes and microbiological systems for the enhancement of leather and related waste, as well as for the production of biobased materials.

The former part of the work involved the use of biotechnological approaches consisting in the selection and use of enzymes and the development of highly effective approaches for obtaining collagen hydrolysates with high added value from tanned waste (shaving and trimming of bovine hides produced with different tanning systems). From a chemical point of view, lether waste is essentially made up of protein material of which collagen represents about 90-95%. In the methods traditionally used to obtain hydrolysates, based on the use of acidic or alkaline hydrolytic agents, the products obtained are normally made up of small, deconstructed polypeptides of about 10-20 kDa, where these characteristics limit their use in numerous fields.

In this context, the former part of the present project has been aimed at enhancing solid wastes from tanning processes, through their conversion into high added value products and, particularly by developing new environmental viable methods for the extraction and reuse of collagen.

The use of enzymes is not new in the tanning sector, but for many years it has been limited to their use in the beamhouse phases (Covington A., et al., 2020); in recent times, their application has been tested, in addition to or in place of chemical approaches for the treatment of waste and for obtaining protein hydrolysates. These hydrolysates, especially if deriving from not yet tanned waste, are used for the production of fertilizers

and biostimulants, as well as, in the presence of given requirements, for the production of food jellies. The treatment of tanned waste is more complex, whereas the variety of currently existing tanning systems makes it difficult to identify effective univocal approaches. The goal of this former part of the work is therefore the development of highly sustainable approaches based on enzymatic and combined treatments (enzymatic / chemical / thermal) for the transformation of these complex substrates. The hydrolysates thus obtained, with desired molecular weight ranges, will then be used, without any modification or cross-linked with casein for different applications, mainly concerning their use in the retanning and finishing phases.

Concerning the finishing phase, given the need to diversify the features of the articles, particularly leather products for the fashion sector, in order to have a wide range of product customization options, biotechnological solutions have also been explored for the creation of bio-based films of a different chemical nature than collagen and its derivatives. More in detail, the production of cellulose films from symbiotic microbiological systems, capable of metabolizing sugars, was tested. This possibility can lead to the development of methods to produce finishing films, through the biotechnological transformation of natural sources of sugars, such as deriving from agri-food waste; in other terms, this approach, in addition to producing a wider range of products for bio-based finishing, would allow to foster virtuous mechanisms of Industrial Symbiosis.

2. Material and Methods

Leather and waste samples

Vegetable tanned bovine shavings, organic tanned bovine shavings, mineral and chromium tanned bovine shavings were collected from tanneries from Veneto District for enzymatic treatments.

Sheep and goat leathers in crust for footwear and leather goods, were selected from Campania District, for finishing applications.

Chemicals and biological reagents

Enzymatic treatments of leather waste: Enzymes were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). NaOH has been used for chemical pre-treatment.

Cellulose film bio-synthesis: Microorganisms symbiotic culture: *Acetobacter xylinum, Saccharomyces cerevisiae* / Culture media of the inoculum: solid plate in 40% distilled water solution, 40% C. sinensis infusion (for 20 min at 80 ° C) and 20% D-glucose; Culture media for biofilm growth: 20% w / v of sucrose, 20% v / v production surplus of vinegar, 40% v / v extract of C. sinensis in infusion.

Main Equipment

MB Line stirred incubators; Rod stirrer 2,200 rpm / 40 l; Nüve Ot 40L; Binder stove model ED SolidLine

Main diagnostic techniques

- *Spectrum One ATR-IR (Attenuated Total Reflection Infrared)* Spectrometer (Frequency range: 4000-600 cm^{-1}).
- *Wild Heerbrugg* Stereoscope equipped with *OPTIKA* Room B3 Digital Camera.
- *Zeiss - Phoni 3* - Optcal Microscope (0.01 - 1 mm - Resolving power: 0,001 mm).
- *Zeiss EVO MA10* Scanning Electron Microscope (Magnification: 10x - 200,000x Resolving power: 2µm) equipped with *INCA X-act ENERGY-250XT* detector.

- Gas-chromatograph *TRACE 1300-THERMO SCIENTIFIC* coupled to a *TSQ 9000-THERMO SCIENTIFIC* mass spectrometer detector and equipped with *ATOM XYZ-TELEDYNE TEKMAR* autosampler for analysis of volatiles on solid and liquid samples.
- *STDQ600 TA* Instrument for DSC/TGA (Differential Scanning Calorimetry/Thermogravimetric Analysis equipped with a *THERMOSTARTM* Mass Spectrometer.
- *FIBRO System AB - Mod. PGX pocket goniometer.*
- *Akta Pure* - Size Exclusion Chromatography (SEC) - *Superose 6 HR 10/300 column/ Gel Filtration Calibration Kit HMW/ Global Life Sciences Solutions, USA LLC*

3. Results and Discussion

3.1 Enzymatic treatment and enhancement of leather waste

Enzymatic approaches for the extraction of collagen have been developed allowing, when compared to the traditional extraction methods, to: *i*) increase the amount of extracted collagen up to 10% (w/w); *ii*) reduce the reaction time (at least two times); *iii*) reduce use of chemical agents up to 10 times; and *iv*) recover a high quality product, thus expanding its range of applicability. The developed enzymatic extraction methods resulted effective on wastes from different types of tanning, regardless of the nature of the tanning agents used.

According to literature, (Godwin Jenifa et al., 2020; Covington A., 2020; Taylor et al., 1996), the most efficient enzymes for collagen extraction from leather solid wastes are alkaline proteases and trypsin. In this work several hydrolytic enzymes were tested, combined with chemical and/or physical pre-treatments.

The recovered collagen has been characterized in terms of protein identification, content of substances of eco-toxicological interest, such as polyphenols and metals, and molecular weights. The diagnostic investigations on the obtained hydrolysate samples confirmed the collagenic nature of the matrix, highlighting the presence of traces of metals and polyphenols, depending on the type of tanning considered. The in-depth analysis using ATR-IR spectroscopy, on the other hand, highlighted a high degree of integrity of the collagen structure: Figure 1, in this sense, highlights how the spectroscopic profiles of collagen extracted by the enzymatic process are comparable to those of the native collagen, suggesting that the extraction process does not affect the structure of collagen, unlike as found downstream of traditional hydrolysis processes (based on the exclusive use of acid or alkaline agents).

The extracted collagen was further characterized by molecular weights and scanning electron microscopy (SEM) analyses, confirming that it preserves an intact structure (Fig.2) and that its quality is such that no subsequent purification step is needed; furthermore, the extracted collagen displays the typical features of native collagen, such as gelling properties. Concerning molecular weights, Enzymatic method allowed to rise from molecular weights up to 6 times.

The use of these hydrolysates, without modifications, or cross-linked with casein has been experimented on a laboratorial scale for retanning and finishing, where collagen cross-linking with casein was optimized through enzymatic mediation, and where further characterizations of the performance of the materials

obtained is under investigation.

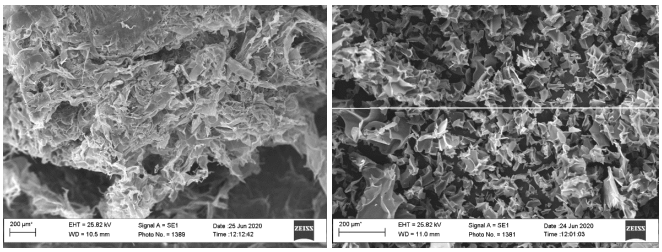


Fig.1: ATR-IR spectra overlap of differently treated and not treated samples

Fig.2: SEM images of native (a) and extracted (b) collagen

3.2 Cellulose film bio-synthesis and application for novel circular finishing

The biofilm, produced by the symbiotic systems described in the materials and methods section, was developed and floated in culture tanks; it was then raised and subjected to 3 successive washes in distilled water inside a beaker under magnetic stirring (each cycle 200 rpm x 10 min). After 20 minutes of partial drying in the oven at 40 ° C, the material was mechanically placed on the surface of the leather; then, subjected to compression by means of a weight of 5 kg for 72 h and conditioned at room temperature for a further 72 h. During the application phase, the film had a pH of 3.5. The reactivity and consequent ability to adhere to leather were correlated, on a chemical level, to the abundance of OH groups, as evidenced by the

ATR-IR examination of the film (Figure 3), which also confirmed the cellulosic nature of the material; on a physical, chemical-physical level, it has been hypothesized that the removal of residual moisture from the film on the tendentially hygroscopic surface of the collagen fibers that make up the leather, in addition to the applied pressure, contributed to a successful application.

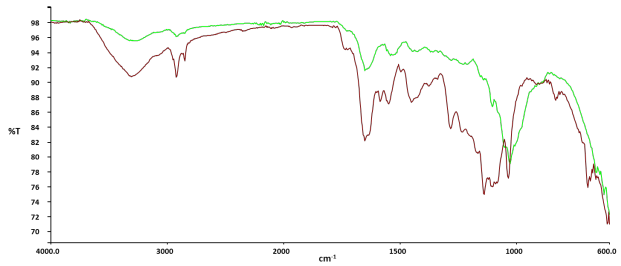


Fig.3: ATR-IR spectra overlap of treated (red) and not treated (green) samples

The film was then characterized by means of optical and electronic SEM microscopy with an X-ray probe, in order to:

- measure the finishing thickness;
- verify the product characteristics;
- verify the absence of metallic contaminants by means of microanalysis.

This investigation highlighted the material's ability to form a thin film, of the order of 20-25 µm, capable of carrying out a protective action, while leaving the original grain structure visible (Figure 4).

In order to verify the protective capacity of the material, against environmental agents, the water penetration time was determined, in accordance with the UNI EN ISO 5403-1: 2012 standard, which was found to be> 30 min, highlighting that the characteristics of water resistance meet the requirements for leathers for footwear and leather goods; the absorption dynamics of water micro-drops (4 µl) were also studied in detail, using the Pocket Goniometer, with the determination of the contact angle in dynamic mode, within 0 and 10 seconds from the impact of the water drops, for the evaluation of the characteristics of surface permeability and hydrophilicity. This test showed that the finishing biofilm, while exhibiting a certain surface hydrophilicity, as can be seen from a CA (Contact Angle) <90°, provides to leather surface a significant resistance to water permeability (Figure 5).

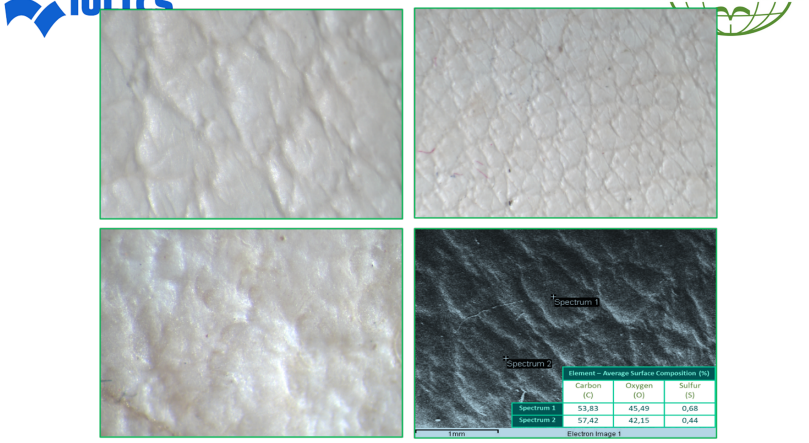


Fig.4: Optical and electronic SEM microscopy analysis of treated samples

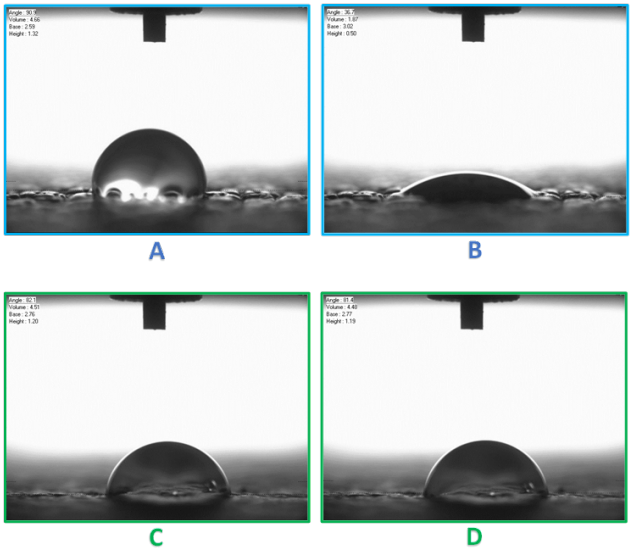


Fig.5: CA value at t_0 (impact time) and at t_{10} (after 10 seconds) of a no treated sample surface (A-B)

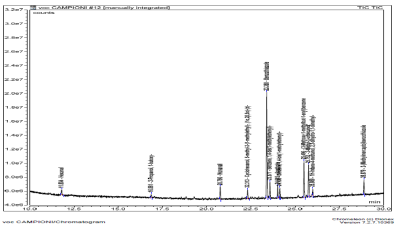
CA value at t_0 (impact time) and at t_{10} (after 10 seconds) of a treated sample surface (C-D)

In order of verifying the solidity characteristics of the material, when used as a new finishing product, its resistance to dry and wet rubbing was also assessed, according to UNI EN ISO 5403-1: 2012; this test has shown that the new material satisfies the dry requirements for footwear and leather goods, while surface abrasions were found after wet rubbing, suggesting the necessity to plan improvements in this sense. In this direction, in-depth studies are underway concerning the use of basecoat from natural non-fossil sources, such as terpenes-based ones deriving from the agri-food sector waste, in order to improve the adhesiveness, as well as the use of natural oils, to be applied as plasticizers, to improve the mechanical properties of the film. The conjugated use of the novel cellulose films and collagen transformation products obtained from activity 3.1 is also under investigation.

The sustainability content of the novel formulation was also highlighted compared to traditional finishing products; the latter, in fact, are essentially made up of polymers in aqueous dispersion, where the use of solvents, diluents, dispersing agents is often required in the formulations, which can sometimes have eco-toxic characteristics. On the other hand, it can be seen, from the comparative analysis of the VOCs (Volatile Organic Compounds) of a sample treated with the bio-film and of an untreated sample, how the biofilm does not produce further volatile emissions, but how, on the contrary, it manages to partially contain the emissions of volatile substances deriving from the leather (Figure 6).

A

Area %	time (min)	Substance	Cas
34.045,592	11.80	Hexanal	66-25-1
20.412,002	16.86	2-Propanol, 1-butoxy-	5131-66-8
58.247,991	20.76	Nonanal	124-19-6
33.063,538	22.31	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1	15356-70-4
515.504,644	23.39	Benzothiazole	95-16-9
66.946,009	23.58	Benzene, 1,4-bis(1-methylethenyl)-	1605-18-1
43.700,529	24.02	Anethole	4180-23-8
38.026,528	24.14	Benzene, 1,4-bis(1-methylethenyl)-	1605-18-1
154.762,890	25.50	(3-Methoxy-1-methylbut-1-enyl)benzene	0
167.540,964	25.75	2-Methyl-1-undecanol	10522-26-6
23.880,736	25.99	1H-Indene-4-methanol, 2,3-dihydro-1,1-dimet	55591-09-8
73.836,817	28.88	2-(Methylmercapto)benzothiazole	615-22-5



B

Area %	time (min)	Substance	Cas
76.586,716	11.81	Hexanal	66-25-1
99.684,604	16.86	2-Propanol, 1-butoxy-	5131-66-8
64.830,259	20.76	Nonanal	124-19-6
141.571,770	22.31	Cyclohexanol, 5-methyl-2-(1-methylethyl)-	15356-70-4
188.938,465	23.39	Benzothiazole	95-16-9
131.087,786	24.02	Anethole	4180-23-8
121.929,707	25.50	1H-Indene-4-methanol, 2,3-dihydro-1,1-dimethyl-	55591-09-8

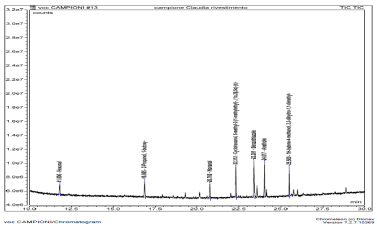


Fig.6: Comparative VOC analysis of a not treated (A) and of a treated (B) sample

4. Conclusion

The enzymatic approaches used for the treatment of tanning waste have been refined for the main categories of shavings treated; categories of enzymes were selected and sequences of enzymatic processes with chemical, chemical and physical pretreatment were identified, depending on the categories treated (organic / vegetable tanning shavings and mineral / chromium tanning shavings); in any case, the processes developed were found to be highly sustainable, allowing the use of traditional chemicals to be minimized up to about 10 times; the treatments were found to be effective in obtaining collagen hydrolysates with technical and structural characteristics to allow their subsequent use in the retanning and finishing phases.

The letter biotechnological approach employed allowed to obtain cellulose films from symbiotic microbiological systems, capable of metabolizing sugars; the obtained films were applied to sheep and goat skins, in order to produce a wider range of products for bio-based finishing; the catheterization of the materials produced made it possible to identify good surface adhesion, protection and impermeability capabilities, and high added value in terms of sustainability of the novel films obtained.

The joint use in finishing of the materials produced by the two approaches will allow to improve the overall quality features of the leather, for the development of novel generations of circular materials with high performance and sustainability.

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